

In vitro studies of specific immunological

unresponsiveness ~~induced~~ in inbred mice

by

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Summary.

The first part of this investigation involved a detailed preliminary study of the mouse mixed lymphocyte culture (MLC) reaction. As a result of these studies it proved possible to adapt the technique for use in microtest plates. The semi-micro assay was shown to provide a quantitative and precise measure of alloantigen-induced lymphocyte transformation and the generation and activity of cytotoxic cells could be clearly demonstrated in the same system. A low incubation temperature was found to be advantageous and the inhibitory effects caused by syngeneic red cells and serum on MLC reactivity as well as the effects of donor pre-immunisation were studied in depth.

The ease of tolerance induction to semi-allogeneic cells in neonatal mice was found to correlate better with the known MLC antigens on these cells than with the known serologically-defined antigens. Adult mice which were demonstrably fully tolerant following such treatment were shown to have no detectable MLC-reactive cells against the tolerising strain in their peripheral lymphoid organs; cytotoxic cells were also absent. Furthermore, serum from such animals was unable to inhibit either the MLC reactivity of normal cells or the cytotoxic activity of immune cells.

By contrast, mice which were given an insufficient dose of cells at birth to induce complete tolerance often rejected their test grafts in a chronic manner, and, in many cases, the lymph node cells of ungrafted littermates were found to possess clear (but subnormal) MLC reactivity.

Finally, an analysis of mice made unresponsive to alloantigens in adult life by use of antigen extracts and anti-lymphocyte serum showed them to possess only slightly subnormal MLC reactivity. Cytotoxic cells were absent but could be induced by in vivo or in vitro immunisation. Although serum blocking activity could not be easily demonstrated in vitro, various lines of evidence suggest a specific and active inhibition of the early stages of immune activation in vivo.

These studies support the notion that self tolerance to major transplantation antigens is due to specific clonal inactivation, whereas partial tolerance induced neonatally or unresponsiveness induced in adult life to the same antigens is apparently the result of an active suppression of immune reactivity.

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Abbreviations.

Ab	=	Antibody
Ag	=	Antigen
ALS	=	Antilymphocyte serum (rabbit anti-mouse)
BDH	=	British Drug Houses Ltd.
Ci	=	Curie
CM	=	Culture medium - RPMI + bicarbonate + antibiotics
CMH	=	Culture medium with HEPES - RPMI + bicarbonate + antibiotics + 15 mM HEPES
CML	=	Cell-mediated lympholysis
cpm	=	Counts per minute
DLN	=	Draining lymph node
FCS	=	Foetal calf serum
FH	=	Ficoll-Hypaque
GVH	=	Graft versus host
HEPES	=	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulphonic acid
I	=	Index of transformation
Ig	=	Immunoglobulin
ip	=	Intraperitoneally
iv	=	Intravenously
LD	=	Lymphocyte-defined
LN	=	Lymph node
m	=	mitomycin-treated
MEM	=	Minimal essential medium
microl.	=	Microlitre
MLC	=	Mixed lymphocyte culture
MLR	=	Mixed lymphocyte reaction
ND	=	Not done
NS	=	Normal saline; not significant
PBS	=	~~Ph~~ osphate-buffered saline
PCA	=	Perchloric acid
PHA	=	Phytohaemagglutinin
RPMI	=	RPMI 1640 medium
SD	=	Serologically-defined
Sp	=	Spleen
SRBC	=	Sheep red blood cells
TdR	=	Thymidine
TCA	=	Trichloroacetic acid
x	=	X-irradiated

CHAPTER 1. Introduction.

The last 10-20 years have seen an explosion of interest in immunology which has been unparalleled in other branches of biology. This interest has resulted from the knowledge that our existence depends on the system of cells which have the task of protecting us from all manner of foreign microparticles, whether living or dead, which penetrate the external defences of our bodies, and that this system has to be so carefully constructed that it can recognise and destroy minute amounts of diverse foreign material while leaving the body's own vast array of constituents unharmed. Furthermore, the possibility of manipulating the immune system so as to provide better protection against particular diseases or to prevent it from attacking the beneficial allografts implanted by surgeons has provided ample incentive for detailed investigations of what has proved to be an extremely complicated biological system.

The years between 1930 and 1960 brought the first real advances in our understanding of transplantation immunology, in particular the recognition that the lymphocyte was the "immunocompetent cell" (for review see reference 1), the importance of surface antigens in determining the fate of transplanted tissues (see reference 2), and the realisation that the rejection of tissues was a result of interaction between immunocompetent cells and surface antigens on the foreign tissues (for review see reference 3). In the early 1950's the discovery of the ability to induce tolerance to foreign tissues by presentation of the antigen in foetal or neonatal life (4,5) provided the incentive for research into possible methods of avoiding allograft or xenograft rejection in man.

One of the greatest advances in our knowledge of the immune system in recent years came with the independent demonstration by workers in America (6) and in Australia (7) of the crucial role of the thymus in the mechanism of graft rejection. Subsequent studies showed that there are two distinct sub-populations of lymphocytes:

(i) those which seem to play a dominant role in the rejection of allografts and which are needed for antibody formation to some but not all antigens;

(ii) those which are the precursor cells for antibody formation to all types of antigen.

The former are produced as a result of differentiation of precursor cells in the thymus and are referred to as T cells, whereas the latter (B cells) are produced in the absence of thymic influences. The importance of the thymus in the ontogeny of the immune system was emphasised in reviews by both groups of workers (8,9).

At about the same time as the discovery of T and B cells chemical analysis of antibody molecules by Porter in England and Edelman in America revealed that they were formed from light and heavy polypeptide chains, the N-terminal ends of which formed the antigen-binding site and accounted for virtually all antibody diversity while the C-terminal ends were of more or less constant composition (10,11) and were responsible for the "biological effects" of antibody such as complement fixation (12).

The 1960's saw the development of in vitro culture systems for the investigation of cellular and humoral immunity. In 1963 Jerne described an in vitro system for measuring antibody-forming cells (13), followed by the description in 1967 of a system in which the entire primary antibody response could be conducted in vitro (14). Similar systems in which cellular immune responses could be studied in culture were described by Bain et al (15), Bach and Hirschhorn (16) and Brunner et al (17). Many of the advances in immunology in recent years, in

particular in the fields of cell cooperation, immunogenetics, and tumour immunology have come from the use of these and similar in vitro systems. In the rest of this chapter those aspects of immunology which impinge on the experimental work described in this thesis will be reviewed in some depth. In all these areas the information gained from lymphocyte cultures has been invaluable to our knowledge of immune processes.

1. The two cell system of lymphocytes.

Perhaps the most important rationalisation of immunological phenomena in recent years has been the demonstration of two major subsets of lymphocytes, those which are referred to as thymus-derived or T-cells, and those which are thymus-independent (B cells), usually referred to as bursa-equivalent or bone-marrow derived lymphocytes in mammals. The distinctive functions of these two major subpopulations of lymphocytes have provided insight into the mechanisms of all types of immune phenomena and no introduction to transplantation immunology would be complete without a discussion of these two types of cells.

(i) Early evidence for the existence of a two-cell system.

Almost 200 years ago Hewson (18) observed that the thymus was filled with cells similar to those found in lymph nodes and in 1900 Beard (19) suggested that the thymus was the origin of the leucocytes that populated the rest of the body. His studies also suggested that the first leucocytes to appear in the embryonic thymus were derived from thymic epithelium, a view which was supported, but not proved, by numerous subsequent studies (see 9). However, studies in the chicken in which male and female embryos were parabiosed have shown that yolk sac stem cells do migrate into the epitheloid thymus and differentiate into lymphocytes there (20), and experiments in which embryonic chick thymuses at various stages of development were cultured in diffusion chambers argued against the ability of epitheloid cells to differentiate into lymphocytes (21).

Similar results have been obtained in the mouse (21). It is thus highly likely that most, if not all, embryonic thymocytes originate from stem cells which migrate into the thymic epithelium. Chromosomal marker studies have shown that in adult animals the thymus continues to be repopulated with immigrant stem cells (9). The differentiation of stem cells into lymphocytes seems to be mediated by hormones released from thymic epithelium (see below).

Although the thymus was long known to be the site of lymphopoiesis, the virtual absence of antibody formation in this organ (22-24) even after prolonged stimulation of the animal (25), coupled with the inability of thymectomy to influence antibody responses significantly in young rabbits (22,26), was puzzling. The latter finding was interpreted as meaning that the thymus has no influence on the immune system (26). Later work (see below) has shown that adult thymectomy can affect immune responses but usually only when immunisation is performed many weeks or months after thymectomy.

It was work in chickens that first suggested the existence of a two-cell system. Glick et al showed that removal of the bursa of Fabricius in the first few days of life reduced the antibody response to an antigen challenge given many weeks later (27). Other workers showed that bursectomy caused some reduction in antibody response when the removal was as late as 5 weeks, but by 10 weeks of age removal of the bursa had no detectable effect on subsequent ability to mount an antibody response (28,29). Mueller et al (29) showed that injection of 19-nortestosterone into 5 day embryonic chickens prevented all bursa development and subsequently hatched chickens were totally unable to make antibody. Unfortunately, treatment of embryos with this hormone also reduces thymus and spleen development so that no clear conclusion can be derived from the hormonal bursectomy experiments.

As mentioned above, thymectomy of young rabbits was found to

have no influence on subsequent antibody formation, and although Fichtelius et al reported in 1961 that thymectomy of young guinea pigs seemed to lower antibody formation (30), the crucial experiments demonstrating the role of the thymus were reported by Miller the same year (7). He found that mice thymectomised in the first 16 hrs after birth had in later life low peripheral blood lymphocyte counts, depleted lymphoid organs, and severely impaired ability to reject skin grafts. His work was very quickly substantiated in a series of papers published by Janković et al (31-33) concerning neonatal thymectomy of rats. In addition to confirming Miller's findings in the mouse, they found that Arthus and delayed skin reactions were reduced in neonatally (first week of life) thymectomised rats, and also that the ability to form antibodies to BSA was extremely low. The effect of thymectomy performed at 2-3 weeks of age was less marked, and, as found by Miller in mice, thymectomy performed later than about 5 days after birth did not reduce the speed of subsequent skin allograft rejection (32). Good's group also reported the effects of neonatal thymectomy in mice and performed similar experiments in rabbits (6). All groups of workers found that neonatally thymectomised mice were subject to wasting disease. That this could be reduced by keeping them in pathogen-free conditions (7) strongly suggested that it was due to a generally high susceptibility of these animals to infection and, by inference, that the thymus played a key role in controlling immunity to infection. An important point in studying neonatally thymectomised mice is to confirm that thymectomy is complete, since even small remnants of thymus can permit considerable immunocompetence to develop (31-33). Failure to check this point caused some misleading results to be reported at first by Good's group (see 34).

(ii) Role of the thymus.

Although the importance of the thymus is most clearly demonstrated by its removal early in ontogenic development, there is no

doubt that despite its continuous involution from birth onwards, it continues to function as a source of lymphocytes long into adult life. Thus, recovery of immune functions following lymphocyte depletion by either X-irradiation (35,36) or treatment with ALS (37,39) in adult rodents was much slower when the animals had also been thymectomised. Adult thymectomy alone can lead to reduction of some lymphocyte functions provided a long period has elapsed between thymectomy and testing (39). These experiments supported the suggestion of Miller (40,41) that certain marrow cells can only achieve their full immunological potential in the presence of the thymus. The question arose, however, as to whether the thymus exerted its influence by releasing a differentiation-inducing hormone or whether residence of stem cells within the thymus was followed by the production and release of lymphocytes.

There is clear evidence that stem cells do enter the thymus in the embryonic chicken (20) and suggestive evidence of this in embryonic mice (21). Furthermore, the entry of stem cells into thymus organs implanted into adult syngeneic mice has been clearly demonstrated (42) as well as of bone-marrow cells into normal thymus (43). Although large numbers of cells die within the thymus, chromosomal marker studies have shown that donor-strain thymus cells can migrate out of thymus grafts into the recipient's tissues (44), and this has been most recently confirmed using θ -allo-antigen surface markers to examine whether cells from CBA thymus implants migrated into the tissues of thymectomised, irradiated, bone-marrow reconstituted AKR mice (45). However, evidence that the emigrant cells from thymus implants were not responsible for the restoration of immunocompetence in thymectomised, irradiated recipients was provided by the experiments of Leuchars et al (44) which showed that H2 incompatible thymus grafts could restore some degree of immune responsiveness to their hosts even though the grafts and presumably the emigrant donor-strain

thymocytes were eventually destroyed. Thus, differentiation of host lymphocytes either in or under the influence of the allogeneic thymus had caused the return of immunocompetence. These experiments were extended by Miller and coworkers (46). They showed that pre-irradiation of implanted thymuses with 500 rads did not inhibit their ability to restore immunocompetence, while pre-irradiation with 2000 rads did. Since both doses of irradiation permitted equally good repopulation of the grafts with recipient cells and good regeneration of lymphocyte levels in the peripheral tissues of the recipients, these authors postulated that the implanted thymuses had the following properties:

(a) the radiation-resistant ability to allow host stem cell proliferation to occur within them to produce immuno-incompetent cells

(b) the radiation-sensitive (at 2000 rads) ability to confer immunocompetence on differentiated but incompetent cells.

The latter function they believed to be due to the action of a hormone released from thymus epithelium.

Very good evidence that the thymus does secrete a hormone conferring immunocompetence on immature populations had been uncovered some years earlier from experiments in which neonatal or embryonic thymus tissue was placed in diffusion chambers which were implanted into thymectomised hosts (47-50). The size of the pores varied from 0.1 to 0.45 microns, and although cell leakage through joinings in the chamber was conceivable, experiments where the possibility of such leakages was examined revealed either none (48) or only occasionally (50) defective chambers. The possibility that the chambers themselves acted as an adjuvant was eliminated by appropriate controls, and although allogeneic thymus was found to restore immune competence to neonatally thymectomised mice as well as syngeneic thymus, neither adult lymph node, neonatal spleen, nor adult spleen was effective (50). Examination of the contents of diffusion chambers at the termination of

the experiment showed only epithelial and reticular cells to be present. The conclusion seems inescapable that thymic tissues can elaborate a hormone or hormones able to induce immunocompetence in immature cells.

Very recently, considerable evidence in favour of the existence of thymus hormonal factors has appeared. Goldstein et al (51) obtained a highly purified protein from bovine thymus which was able to re-induce azathioprine-sensitive SRBC-rosetting lymphocytes when injected into adult thymectomised mice. Such cells are lost within a few days of adult thymectomy in mice, and evidence has been presented that they are a sub-population of thymus-derived cells (52). Their regeneration occurred rapidly (less than 24 hrs), indicating that the induction of these cells was not dependent on division of precursor cells.

Thymus extract prepared in this way was shown in a series of elegant experiments by Komuro and Boyse to induce thymus cell-surface antigenic markers (TL and Θ) in fractions of spleen and bone-marrow cells lacking these antigens before treatment (53). Incubation of such cells with mouse thymus extract for as little as 1 hr induced detectable amounts of TL antigen on their surface, and whereas extract prepared from thymic epithelium was active, extracts from thymus lymphocytes or whole spleen were inactive. As might be expected, cycloheximide inhibited the antigenic conversion which was subsequently shown to occur with cells from 14-day embryonic liver or from nu/nu mice (54). Extracts of human thymus prepared in the same way were able to induce surface antigens characteristic of T cells when incubated with appropriate fractions of bone-marrow for 2-3 hrs. However, no PHA or MLC-responsive cells were produced (55). Very recent work has shown that the antigenic conversion properties of thymic extracts are attributable to two closely related peptides of

about 7000 daltons which are active at extremely low concentrations (56).

It is noteworthy that studies of antigenic conversion of mouse bone-marrow cells by thymus extracts has shown the appearance of TL antigen (53,54,56). Since thymocytes bearing the TL antigen do not leave the thymus (57) and are not immunologically competent (58 and see below) further differentiation is necessary to produce competent cells. That thymus extracts are alone incapable of inducing immunocompetence in bone-marrow cells is shown by the in vitro studies of Trainin's group (59,60), the initial differentiation stage of bone-marrow cells occurring after activation of the cyclic AMP system (61). The participation of other hormones in the induction of full immunocompetence is a real possibility, and some workers have claimed that growth hormone may be involved (62,63).

(iii) The roles of B and T cells.

The importance of the thymus for the normal functioning of the immune system has been emphasised at length in the preceding section. Full immune competence, however, also requires the presence of a second population of lymphocytes which are non-thymus-dependent. In birds this second population is bursa-dependent, removal of the bursa at hatching greatly reducing the ability of chickens to form antibodies in adult life (27-29; 64). The bursa is similar to the thymus in having very large numbers of DNA-synthesising cells (65) and being immunologically inert, the incidence of antibody-forming cells being extremely low in this organ (65,66). Although removal of the thymus at birth reduces transplantation immunity in chickens (67) antibody responses are inconsistently affected (64). By contrast, removal of the bursa at the same time has relatively little effect on graft rejection times (67). These and many other studies on the comparative effects of thymectomy and bursectomy in chickens led Warner and co-workers to postulate that there existed a partition of immunological

function between thymus-dependent and bursa-dependent cells, the former being responsible for transplantation immunity and delayed hypersensitivity reactions, the latter for the actual production of antibody (68). Studies where irradiation was used to amplify the effects of organ extirpation (69) fully confirmed the earlier evidence for a two cell system in birds, and conclusive evidence that the bursa is essential for the generation of immunoglobulin-producing cells was provided by the demonstration that embryonic bursectomy would in at least a proportion of cases cause complete agammaglobulinaemia and an absence of germinal centres and plasma cells from the peripheral tissues (70).

The similarity of the effects of thymectomy in birds and mammals (see above) hinted that the mammalian lymphocyte system was also divided into two sub-populations of cells analogous to those demonstrated in chickens. Further evidence for this came from histological studies of the lymphoid organs of neonatally thymectomised mice which showed that thymus-dependent cells occupied one part of these organs, the remaining areas being occupied by a thymus-independent population (71). At about the same time, studies with chromosomal markers demonstrated that thymus-derived cells did not participate directly in antibody synthesis (72).

(Evidence for the bursa-equivalent organ in mammals has been conflicting, the gastrointestinal tract, appendix, tonsil, Peyer's patches, and haematopoietic tissue having been proposed at various times for this role. Recent work has provided convincing evidence that in the embryonic mouse the liver acts as an initial source of immunoglobulin-bearing cells (73), but these experiments did not exclude the possibility that other sites might participate in B cell maturation).

Various methods were subsequently employed to study the functions of thymus-dependent and thymus-independent populations in

mice. The major line of attack has been the development of antisera against membrane antigens specific for T or B cells. T cells can be identified by the presence on their surface of θ (74,75). Precautions must be taken with the preparation of anti- θ antisera (76) since auto-antibodies can be present, and in cytotoxic tests the type of complement used seems to be important if high specificity is to be obtained (77). Interestingly, guinea pig serum, usually used as the complement source, seems to have natural antibodies against mouse cells, particularly thymocytes (78). Thymocytes possess a distinctive antigen, TL, in some strains of mice which is not present on peripheral T cells (57). B cells can be readily identified by the presence of surface immunoglobulin (79,80) or mouse-specific B lymphocyte antigen (76). Recently, a surface antigen specific for plasma cells has been identified (81). The sum of Ig-positive and θ -positive lymphocytes in spleen and lymph node is usually close to 100%. That minor populations of other cell types exist, however, is suggested by recent studies indicating that the cells mediating antibody-dependent lymphocytotoxicity (82) bear neither surface marker (83).

Attempts have been made to extend these methods to other species of mammals, most importantly to humans. Identification of immunoglobulin-bearing cells is fairly straight-forward, but problems have been encountered in obtaining antisera specific for thymus-derived cells. The use of an alternative marker for thymus-derived cells in humans, spontaneous rosette formation with sheep red blood cells, has therefore recently been explored, particularly by Bach's group (84), with promising results. Strong evidence for the existence of a B and T cell system in man has come from studies of immunodeficiency diseases (85).

A second method of analysis of B and T cell function has been the use of cell separation techniques based on (a) the presence of surface

immunoglobulin on B cells (86,87), (b) the presence of Fc and/or Ig receptors on B cells (88,89), (c) difference in electric charge between B and T cells (90), (d) the affinity of B cells for nylon fibres (91), or (e) the presence of any surface marker that can combine with fluorescent antibody (92). Separation techniques are proving increasingly valuable as a means of assessing which cell types may mediate a given function in vivo or in unseparated lymphoid populations in vitro. However, in order to draw valid conclusions concerning the presence or absence of a certain functional activity in a purified sub-population it is essential to have high yields of cells after separation and to be able to demonstrate with cell markers or mitogens (see below) that removal of other cell types has been complete.

In view of the difficulties encountered in producing antisera specific for human lymphocyte sub-populations, the possibility of using mitogens as a means of identification has been explored. There is good evidence that under normal conditions PHA is a specific mitogen for thymus-derived cells in the mouse (93,94), even though both B and T cells can bind PHA (95) and B cells can respond when the mitogen is coupled to Sepharose beads (96). Similarly, bacterial lipopolysaccharide stimulates only B cells (94). PHA has been used for a long time as a means of assessing the general immunocompetence of human lymphocyte populations, but recently some conflict has arisen as to whether it is truly specific for human T cells (97,98). Sheep cell rosettes may well provide the best marker for T cells in man at the present time (84), but as pointed out by Greaves in a recent review (99), it is really essential to use a combination of tests rather than relying on any single one. Furthermore, the use of PHA or SRBC as tests for T cells in disease states may give extremely misleading results due to:

(a) selective loss of lymphocyte sub-populations during

separation of leucocytes from peripheral blood (99)

(b) changes in total lymphocyte number rather than in the relative proportions of T and non-T cells

(c) selective loss of a small subclass of T cells would not be detected. Ideally, tests for specific antigen-reactive cells are needed.

The techniques described have allowed the identification of cells participating in various kinds of immune reactions. At the moment the only clearly identified role for B cells is as precursors for antibody-secreting cells. After injection of antigen into the animal part of the material is found to localise on the surface of dendritic macrophages in the draining lymphoid organs (100, 101), and these are believed to present antigen to B cells. Evidence that macrophages are important as presenters of antigen to B cells has also been suggested by in vitro studies (102,103), and these will be described in more detail below. Following interaction with the macrophage-bound antigen a series of divisions among the precursor B cells leads to the production of plasma cells (104). The virgin B cells apparently have IgM receptors (105) which can switch after antigenic stimulation to IgG (106). That this switch requires only an interchange of C-region genes was suggested by studies showing that anti-idiotypic antibodies reacting with IgG molecules late after immunisation also react with IgM molecules from earlier bleeds (107), and it is certainly compatible with genetic studies which have shown close linkage between C and V region genes in the germ line (108). The detection of cells with IgM on their surface and IgG molecules inside (109) supports the idea that C-region switches can occur in an antibody synthesising cell.

T cells have been established as the principal effector cells in various aspects of cellular immune responses. Their importance in the allograft reaction is shown by the inability of thymectomised mice to

reject skin grafts (see above) and their roles in mediating delayed hypersensitivity reactions (110), in protecting animals against infection (111), and in providing the effector cells in certain *in vitro* cytotoxic reactions (112) are now generally accepted. They also play a crucial role as "helper cells" during antibody formation to most, but not all (113,114), antigens, and this aspect of their function will now be discussed briefly.

Although studies with hapten-carrier complexes had for a number of years hinted at the possibility of two types of cells cooperating in the immune response, the clearest demonstration that different cells recognised the carrier and hapten parts of conjugates came from the experiments of Mitchison (115), who demonstrated in transfer experiments in mice that unless cells were primed to the correct carrier no secondary response could be induced to the hapten. Raff showed subsequently that the carrier-primed cells were in fact T cells (116), strongly suggesting an analogy between Mitchison's experiments and those performed several years earlier by Claman et al (117,118) in which thymocytes and bone-marrow cells were found to act synergistically in restoring antibody responses to irradiated mice. These and similar experiments could be explained by postulating that either T and B cells interact directly, T cells acting to 'focus' haptenic determinants onto B cells (119), or that a substance was produced by antigen-stimulated T cells which was able to confer competence on the B cell population.

Strong evidence in support of the latter hypothesis was presented by Feldmann and Basten (120,121), who demonstrated that T-B cooperation could occur when the two sub-populations were separated by a cell-impermeable membrane. *In vitro* studies also showed that macrophages were essential for T-B cooperation (102,103), but not for antibody production to thymus-independent antigens (103): the macrophages apparently bind an antigen-containing factor released from antigen-

treated T cells and present it to the B cell population (122). There is evidence to suggest that this factor is a complex of antigen and T cell surface immunoglobulin receptor (122,123), this latter having, at least in the uncomplexed state, a high affinity for macrophage membranes (124). Although the weight of evidence is in favour of T cells having immunoglobulin receptors (124,125), there are lines of argument to suggest that T cells have a non-immunoglobulin receptor (126). As pointed out by Raff recently (127), it may be that T cells have two types of molecule involved in antigen recognition, and clearly the validity of Feldmann's model depends on T cells having at least an immunological receptor.

An alternative view of cooperation is that activated T cells secrete a non-specific factor which allows B cells to respond to antigen. Although there is clear evidence that such factors exist (128-130) they are unable on their own to account for the specificity of the carrier in vivo (131). The most likely explanation is that such factors augment a specifically induced response, and indeed there is evidence from two in vitro systems that specific and non-specific factors act together (128,130).

There can now be no doubt that the T cell sub-population of lymphocytes plays a multitude of roles in various immune functions, providing helper cells for antibody formation, initiator and effector cells against alloantigens, memory cells, and probably also suppressor cells. It is therefore not surprising that there is a growing body of evidence supporting the existence of subclasses of T cells.

For instance, it has been found that T cells at different stages of maturation possess different surface antigens. Thus, TL -ve, θ -ve blasts which enter the foetal mouse thymus differentiate over the following few days into TL +ve (in strains possessing the TL

gene), Θ +ve thymocytes (45). Furthermore, in comparison to thymocytes, peripheral T lymphocytes have increased amounts of H-2 antigen (132), decreased amounts of Θ (45,132), and TL is lost completely (132). However, within the thymus there is a sub-population of cells representing 3-5% of total thymocytes which are relatively resistant to corticosteroids and radiation (133) and which have many of the characteristics of peripheral T cells including similar antigenic make-up (58), ability to respond to allogeneic cells and PHA in vitro (134), and general immunocompetence (133,135). Evidence that these cells are not derived from recirculating peripheral blood cells comes from studies of chromosomally marked thymus grafts implanted into syngeneic animals (136) and from the finding that ALS treatment depletes the pool of recirculating T cells but does not affect the immunocompetent thymus cells (137). Furthermore, the cortisone-resistant cells seem to be located in the medulla (138), and histological studies indicate that there is a transport of cells from the thymus cortex to the medulla (139,140).

The TL -ve, cortisone-resistant thymic cells have been found to be largely lymph node seeking, recirculating lymphocytes in homing studies (141,142), whereas the TL +ve cells are mainly spleen seeking (141) in which organ they rapidly lose their TL antigens (141). Studies of GVH reactions have shown that two types of T cell cooperate (143,144) and evidence has been presented that one of these cells (T1), found mainly in spleen and thymus, is relatively resistant to ALS but is eliminated by neonatal thymectomy, whereas the second, recirculating cell type (T2), found mainly in lymph node, is more susceptible to ALS than T1 and not completely eliminated by thymectomy (144). These experiments were far from clear-cut and much more convincing evidence for the existence of T cell subsets has been provided by the studies of Bach et al (52) who showed that sheep cell rosettes formed by T cells from mouse lymph

node had a lower sensitivity to azathioprine than thymus and spleen rosettes. Rosette-forming cells with high sensitivity to azathioprine (T1) were lost from the spleen within 6 days of adult thymectomy but were considerably less sensitive to ALS than the rosette-forming cells of lymph nodes (T2). The rapid reappearance of T1 cells after thymus grafting may mean that these cells are not short-lived but require the presence of thymic hormone(s) to function (51, 52).

These experiments have shown that there are at least two subclasses of T cells: one (T1) which homes from thymus to spleen and which is dependent on the presence of the thymus to function normally; the other (T2) which homes preferentially to lymph nodes and probably recirculates but does not require the continuing presence of the thymus to function. The latter cells have lower amounts of θ antigen and are more susceptible to ALS in vivo than the former (52).

Although little is known about the function of these cells it may well be that they are responsible for some of the diverse roles of T cells which have now been recognised. A number of workers have recently suggested that different T cell subclasses are involved in MLC and PHA responses (145-147), in cytotoxic and helper functions (148,149), in MLC and GVH reactions (150), and in MLC and cytotoxic reactions (151), but at the moment none of the reports is wholly convincing. The field is likely to become more complex over the coming years, and already there is evidence for up to 5 stages of T cell maturation in the mouse (152), as well as of subclasses of B cells (153,154) and of peritoneal macrophages (155,156). It is only to be hoped that rationalisation of data will rapidly follow its accumulation in this field.

2. Graft rejection.

(i) Sensitisation to allografts.

As will be discussed below (Section iii), after implantation of a skin graft blast cells appear in the draining nodes, cytotoxic cells are produced there, and cellular infiltration of the grafts occurs. One of the problems of transplantation immunity, however, has been the mechanism by which the sensitisation process, which causes these various changes and ultimately leads to rejection of the graft, takes place. Thanks largely to the work of Billingham and co-workers it has now been clearly demonstrated that drainage of the skin graft site by lymphatic connections is essential for sensitisation to occur. Thus if skin grafts are implanted in sites lacking lymphatic drainage such as on hamster cheek pouch skin (157), skin flaps on the flanks of guinea pigs supplied by an "umbilical cord" containing blood vessels but not lymphatics (158), or in the centre of large graft beds (159), they are not rejected and do not elicit a state of sensitisation in the recipient; yet they are destroyed if the recipients have been presensitised, demonstrating that effector cells can gain free access to the grafts via the bloodstream. Although sensitisation can eventually occur in the absence of draining lymphatics (160), these vessels are clearly important for the normal induction of an immune response to skin allografts.

With kidney grafts, on the other hand, lymphatic drainage seems to be unimportant for the induction of sensitisation. Thus, in experiments where care was taken to avoid lymphatic connections occurring with the grafted organ, sensitisation occurred more or less normally (161,162). Indeed, when F_1 kidneys are perfused with thoracic duct lymphocytes from the parental strain for 1 hr, the perfusate can transfer sensitivity to a syngeneic host (163), clearly showing that lymphatic connections are unnecessary.

In 1958 Medawar postulated that sensitisation to grafts may occur peripherally, lymphocytes confronting antigen within the graft rather than in the draining node (3). In the case of skin grafts, vascular connections are not made until about 2 days after grafting, and it appears that sensitisation primarily occurs following the passage of graft antigens down the draining lymphatic vessels. Thus, the experiments of Billingham and colleagues (157,159) demonstrated that even after vascularisation has occurred sensitisation of the recipient is not possible unless there are lymphatic connections. * Furthermore, although the great majority of lymphocytes found within an allogeneic skin graft are the products of recent cell division (164) little cell multiplication seems to occur within the graft (165). Histological studies have shown that very few cells are present within the graft before day 5, the large number of cells present thereafter apparently reaching the graft via the newly formed vascular connections and inflicting damage soon after their arrival (166,167). These various findings strongly argue against sensitisation occurring within the graft. However, it is possible that lymphocytes could confront antigen at the graft-host interface and then travel down the lymphatic vessels to the draining node. That such a mechanism is feasible is shown by the studies of Hall and Morris (168,169), who found that under normal conditions significant numbers of lymphocytes do leave the blood vascular system, perfuse through the outerlying tissues, then percolate down the afferent lymphatics.

By contrast, in organs that are connected with the host vascular system at the time of grafting there is evidence that sensitisation may occur within the grafted organ. Strober and Gowans (163) found that, whereas the perfusate obtained by passing thoracic duct cells through allogeneic kidneys transferred sensitivity to normal rats, cell-free perfusate was unable to do so. In an extensive study of renal allografts in sheep, Pedersen and Morris (170) found that 2-3

days after grafting large numbers of blast cells began to appear in the lymph which drained from the grafted kidney. Cells obtained during the first day of cannulation were not transformed, but after culture in vitro for 2-3 days they did transform, and when injected into distal host lymph nodes they induced significant cellular proliferation and antibody production clearly showing that these cells had either picked up antigen on their surface or had been committed to transformation after coming into contact with antigen in the graft. Furthermore, when all draining lymph was removed from the animals, little reaction to the allograft occurred in the draining node, yet graft rejection occurred in the normal time, blood vessels becoming blocked with blast cells. Subsequently it was reported that blast cells were also present in the afferent lymph draining from allografted kidneys in both man (171) and in dogs (171,172). Interestingly, in humans, blast cells appeared despite immunosuppression and in patients whose kidneys were not rejected during the follow-up period of 12-18 months (171). There was also the suggestion that the incidence of blast cells in the draining lymph correlated with the degree of donor-recipient incompatibility as judged by pre-transplant MLC tests. These findings (163,170-2) strongly argue in favour of peripheral sensitisation occurring in kidney allografts.

Regardless of the site at which sensitisation occurs, an important question is the nature of the antigenic material that induces the allograft response. That whole cells as opposed to antigenic fragments must pass through the afferent lymphatics draining a skin graft was suggested by the finding that skin grafts fail to sensitise their hosts if separated from them by a cell-impermeable membrane (173). Although this experiment can be interpreted in numerous ways, one explanation is that only cell-bound antigen from skin grafts is able to induce sensitisation. Recently it has been shown that A

strain skin grafts placed on (C57 x A) F_1 mice are able to induce a graft-versus-host reaction in the draining lymph nodes of the host (174), providing strong evidence that not only do skin grafts contain significant numbers of passenger-leucocytes but that these cells are able to travel down the afferent lymphatics to the local lymph node.

These experiments are in line with the numerous claims that skin and organ grafts contain significant numbers of passenger leucocytes that are able to induce a state of sensitisation in recipient animals. Thus, Steinmuller (175) showed that A strain skin which had been grafted onto chimaeric A strain mice made tolerant to C3H by injection of (C3H x A) F_1 cells at birth was able to sensitise normal A strain mice to C3H skin taken from the same tolerant donors. Similarly, skin from CBA mice irradiated and reconstituted with CBA spleen cells caused greater sensitisation of C3H recipients to subsequent CBA grafts than did skin from irradiated CBA mice reconstituted with C3H cells; and whereas skin from C3H mice reconstituted with C3H spleen cells caused no sensitisation of C3H recipients, skin from mice reconstituted with CBA cells did (176). In these experiments sensitising grafts removed even as early as 2 or 3 days after implantation were able to sensitise recipients if they carried the appropriate passenger cells, indicating that these cells could make contact with the host's immune system before vascular anastomoses were established.

Similar experiments have shown that passenger leucocytes in transplanted kidneys can induce sensitisation in recipients (177), and there are many reports that treatment of donors with high doses of immunosuppressive drugs can delay the onset of allograft responses in recipients. Although these experiments can be criticised on the grounds that immunosuppressive drugs gain access to the recipient, the fact

that irradiation (178,179) or perfusion (180) of grafts prior to transplantation works equally well if not better supports the notion that passenger leucocytes do play a role in the normal sensitisation process. Exactly how important their role is is not clear at the moment. Recent experiments have shown that skin allografts lacking allogeneic passenger leucocytes are unable to sensitise the recipient if removed within 48 hrs, whereas they cause strong sensitisation when left in place for 7 days (179). It seems that passenger leucocytes in skin may merely accelerate the allograft response by providing an early source of graft-strain antigen to the host. However, the situation with other types of graft may be different since sensitisation, at least to kidney grafts, occurs peripherally (see above) and, whereas epidermal cells are able to stimulate in MLC (181-185), kidney cells are unable to do so (181,182). It could well be, therefore, that passenger leucocytes in organ grafts provide an important source of immunising antigen. Alternatively, it has been shown that parental strain kidneys placed in F_1 recipients can be damaged by GVH reaction if they have been injected with 'passenger' lymphocytes (186). Thus, by removing passenger lymphocytes one may in part be avoiding non-specific damage, with the possibility of increased antigen release, caused by an intra-organ GVH reaction.

(ii) The skin graft as a model for graft rejection.

Because of the ease with which skin can be grafted orthotopically and observed thereafter, much of our knowledge of the mechanisms of graft rejection has come from studies of the fate of skin allografts. It appears in fact that skin allografts are more sensitive to the actions of the hosts' immune system than other organs (187-191), and although rejection of other organs may require some differences in the marshalling of the hosts' immune system there is no evidence that the mechanisms involved in the rejection of skin grafts are in any way exceptional, and skin allograft rejection can

still be taken as a model for the induction of transplantation immunity.

(iii) The roles of cells and antibody in mediating graft destruction.

Since the experiments of Mitchison (192) and Billingham et al (193) in the early 50's demonstrated that immunity induced by skin grafts could be transferred from one mouse to another with cells but not with serum, the balance of evidence has always favoured the direct participation of cells rather than antibody in the destruction of allografts. However, the possibility that antibody does play a crucial role in graft rejection can even now not be ruled out.

Thus, it can be argued that the adoptive transfer of sensitised cells merely transfers antibody-forming cells to the secondary recipient, and the failure of serum to transfer immunity could be explained by either an insufficient quantity or type of antibody at the time when serum was removed from the donor, or injection of insufficient amounts of immune serum or injection at the wrong time in relation to grafting in the secondary recipient. The fact that serum antibody is easily detectable at the time of graft rejection makes it extremely difficult to prove that it is not important, and indeed, studies of the incidence of plaque-forming cells in the draining lymph nodes and spleens of mice transplanted with H2 incompatible skin demonstrated their presence at (194) or before (195) the onset of graft damage.

One method of attacking the problem is to examine what kind of cells are involved in allograft responses. Using the graft-versus-host (GVH) reaction as an analogue for host-versus-graft reactions, the importance of the small lymphocyte in inducing allograft responses was implied by the high reactivity of thoracic duct cells (196,197) which are rich in small lymphocytes. That the small lymphocyte was indeed the inducing cell was conclusively demonstrated by

induction of GVH reactions with purified thoracic duct cells containing less than 0.05% small lymphocytes (197). After injection of thoracic duct parental-strain cells into F_1 hosts large pyroninophilic blast cells were seen to develop (197), and in contrast to the recirculation of thoracic duct lymphocytes observed in a syngeneic transfusion (198), parental cells could not be removed from F_1 recipients by cannulation of the thoracic duct (197).

Similar blast cells were subsequently found in the lymph nodes draining skin allografts (199,200), and in cultures of lymphocytes stimulated by either PHA (201) or allogeneic cells (15,16). Unfortunately, the precursors of antibody-forming cells may have a similar ultrastructure (202), so the observations of cell morphology provide no evidence either for or against participation of antibody in the normal allograft response.

A second approach has been to study the nature of cells infiltrating grafts. Numerous histological studies have shown that little infiltration of skin grafts occurs until about day 5 when there is a sudden inflow of lymphocytes, monocytes, and histiocytes (166,167,203). This inflow of cells precedes any detectable destruction of graft tissue and, in a detailed electron microscope study, Wiener et al (167) observed a close interdigitating association between many of the invading cells and graft cells and only rarely found cell destruction occurring in regions of the graft free from infiltrating cells. These authors also found only rare plasma cells in grafts, the few that did appear arrived only when graft destruction was well advanced. Using enzymic procedures to digest skin grafts which had resided on allogeneic mice for varying periods, Jakobisiak (203) found that the majority of invading cells were lymphocytes, most of the remaining cells being macrophages, and only 0.02% of immigrant cells were plasma cells. Waksman's study of rat

allografts (166) suggested that the death of the graft followed severe destruction of blood vessels at the base of the graft by invading mononuclear cells, the graft undergoing subsequent ischaemic damage.

Although the absence of plasma cells does not mean that antibody-synthesising cells are absent (204,205), the fact that invasion of grafts by host cells precedes any visible sign of destruction, and that the invading cells are closely associated with lesions in the graft tissue (167) or in blood vessels (166), provides strong circumstantial evidence that the infiltrating cells are in fact aggressor cells. Further circumstantial evidence that cells rather than serum antibodies are the effectors of graft destruction came from the demonstration that lesions resembling hypersensitivity reactions could be induced by injection of donor antigen extract into guinea pigs sensitised by skin grafting (206). A similar reaction could be provoked in donor animals by injecting sensitised cells but not serum from sensitised animals.

Further evidence in support of a direct reaction by lymphoid cells against allogenic tissues was obtained by Elkins (207,208) who showed that when parental strain cells were injected under the kidney capsules of F_1 kidneys a local GVH reaction occurred in which destruction of cortical tubules was seen only where infiltrating mononuclear cells had intimately surrounded a tubule.

However, the strongest evidence in support of a direct destructive action of host lymphocytes against foreign tissues has come from (a) systems where humoral and cellular responses can be clearly dissociated (b) in vitro studies.

The former type of evidence is based upon the clear dissociation between thymus-dependent functions and thymus-independent functions in birds and mammals. The two cell system has been

discussed in some detail in part 1 of this chapter. The clearest demonstration of the fact that graft rejection does not depend on antibody formation in vivo comes from the work of Cooper et al (69) in chickens. They amplified the effect of neonatal bursectomy and thymectomy by inactivating any already mature lymphocytes with sub-lethal X-irradiation. Removal of the thymus from neonates followed by whole body irradiation caused a strong inhibition of their ability to mount delayed hypersensitivity reactions and reject skin grafts, and of their cells to induce GVH reactions. By contrast, bursectomy followed by irradiation led to agammaglobulinaemia and a total inability to make antibody; yet these birds could still reject skin grafts and their cells could mount good GVH reactions. Interestingly, their ability to reject skin grafts was slightly diminished compared to irradiated controls, suggesting that although graft rejection is highly T cell dependent, B cells and/or antibody may play some minor role.

In mammals such a direct approach is not possible, since the B cell system cannot be selectively destroyed. However, studies of congenital agammaglobulinaemia in man, a condition in which there is an absence of plasma cells and extremely low serum immunoglobulin levels, have shown there to be a more or less normal ability to reject allografts (209). Similarly, foetal sheep maintained in a completely agammaglobulinaemic state can reject skin allografts (210). Further results which support these findings have been obtained by Elkins and Palm who found that although double skin-grafting of Lewis rats with Fischer skin induced no detectable haemagglutinating antibodies; the grafts were rapidly rejected with an MST of 9 days (211). Subsequently, Lance and Batchelor reported that mice given weekly injections of ALS retained H2 incompatible skin grafts indefinitely, yet many such mice had antibody titres comparable to those found in non-ALS treated mice which had rejected their skin grafts normally (212).

Others have found the same effect, i.e., retention of grafts in situations where there is a clear humoral response. Boyse and colleagues have reported three examples of this kind:

(a) Irradiated mice reconstituted with (C57 x A) F_1 bone-marrow cells often fail to reject C57 skin (213,214) yet those mice which retain C57 skin grafts produced similar amounts of anti-Sk.2 antibodies to mice which reject their skin grafts (214).

(b) Certain strains of female mice (e.g., C3H, DBA/2) rarely reject skin grafts from syngeneic males, whereas in other strains (e.g., C57Bl/6) all females reject male skin. Yet Goldberg et al found that 27/27 C3H females and 5/7 DBA/2 females that failed to reject male skin grafts produced similar amounts of anti-H.Y antibody as 10/10 C57Bl/6 females which rejected male skin (215).

(c) A/ θ .C3H mice rarely reject congenic A/ θ .AKR skin yet most such recipients produce high titres of cytotoxic anti- θ .AKR antibodies. In the reverse direction (θ .C3H skin on θ .AKR mice) grafts are usually rejected but production of anti- θ .C3H antibody is transient and barely detectable (216).

These results taken together strongly suggested that thymus-derived lymphocytes were responsible not only for the induction of allograft immunity but also that they provided effector cells for the destruction of skin allografts. In recent years considerable further evidence to support this hypothesis has emerged from in vitro studies of the behaviour of lymphocytes from either normal or pre-sensitised animals when confronted with allogeneic stimulator or target cells in vitro. An in vitro model for both inductive and effector phases of the allograft reaction is provided by the mixed lymphocyte culture reaction. There is now no doubt that the responder cells in MLC reactions are T cells and that pure suspensions of T cells (in the presence of small numbers of macrophages) can not

only transform when confronted with allogeneic stimulator cells but also differentiate into killer T cells, which cause rapid lysis of appropriate target cells (for a discussion of the role of T cells in the MLC reaction see the introduction to chapter 3). In vitro studies have also demonstrated that cytotoxic cells are generated after in vivo sensitisation of animals with allogeneic cells, skin grafts, or organ grafts.

The first recorded observation of cytotoxic cells in allografted animals was by Govaerts (217), who showed that thoracic duct cells from kidney allografted dogs were cytotoxic to the donor's kidney cells in vitro in the presence of immune serum. Although immune serum on its own was not toxic to the cells, the experiments were far from clear-cut, and could not be validly interpreted as demonstrating cytotoxic cells. A clearer demonstration of the existence of cytotoxic cells was provided by Rosenau and Moon (218) who took spleen cells from Balb/c mice multiply immunised with L cells (C3H fibroblast line). Unlike normal spleen cells they were observed to cluster around L cells in vitro and after a time the target cells withdrew their processes, rounded up, and were observed to disintegrate. Cell counts performed after incubation verified that target cell destruction had occurred, and L cells incubated with the sensitised cells did not stain with fluorescent anti-mouse-immunoglobulin sera. Various workers subsequently reported that sensitised but not normal cells strongly adhered to specific target cells in culture (219-226), agglutination of large numbers of sensitised cells around target cells being reported in one case (219). The most detailed morphological study was by Rosenau (220) using time-lapse cinematography. He reported that after lymphocyte attachment to target cells they rounded up and after a violent movement

of cytoplasm all mobility in the target cells ceased. They then swelled up and lysed. The great majority of attached lymphocytes also lost their mobility at this time and were presumably dead. That the cytotoxicity caused by sensitised cells was due to direct killing by the cells rather than to the intervention of antibodies was strongly suggested by these morphological observations and the fact that addition of complement had either no effect (220,221) or only a minor one (217,225) on the degree of cytotoxicity, and that immune serum in the absence of complement was ineffective either alone (217-219,223) or in the presence of normal cells (220,225). Furthermore, cells killed by either heating or freezing and thawing were without effect (224,226), as were supernatants from cultured immune cells (220,224). When target cells and immune cells were separated by Millipore membranes no cytotoxicity occurred (220,224).

In these experiments fibroblasts were usually used as the targets, the degree of reaction being evaluated by cell counting after 24 or 48 hrs incubation. The long incubation period, and the fact that no killing was usually detectable before 24 hrs left open the possibility that sensitisation was occurring in the cultures. Furthermore, since the target cells multiplied during this period a decrease in the number of target cells could be caused by either mitotic inhibition or detachment from the culture surface. Although in some studies these objections were shown to be invalid by using non-dividing target cells (223,224) or by directly observing cell lysis (220), it was highly desirable to use a rapid assay and one where target cell lysis could be directly monitored. The former aim was initially achieved by the use of plaque-forming cells as targets, these being extremely sensitive to the presence of cytotoxic cells (227), and subsequently by use of a cloning assay (228) which achieved high sensitivity because the subsequent multiplication of target

tumour cells was extremely susceptible to even the mildest damage inflicted by cytotoxic cells. The latter aim was achieved by the labelling of internal cell components with radioactive precursors. The method was first employed by Perlmann and Broberger (225), but the P-32 and C-14 labels that they used had very high spontaneous release rates. Meanwhile, Wigzell demonstrated the great advantage of Cr-51 for cytotoxic reactions (229) and the use of this label was elegantly exploited by Brunner and colleagues (17) to evolve a cytotoxic assay based on the exquisite sensitivity of P-815-X2 DBA/2 mastocytoma cells to lysis after membrane damage.

Previous studies with the long term cell counting assay (224) and the more sensitive cloning assay (228) had shown that the logarithm of target cell survival decreased linearly as the number of added effector cells increased from zero up to quite high effector: target cell ratios. Such kinetics indicated that a single effector cell was able to kill and lyse a single target cell. The question arose as to what the cell type was. The first evidence indicating that it was a T cell came from studies of cytotoxic and plaque-forming cells found in the spleens of lethally irradiated mice in which a GVH reaction had been induced (230). If the donor inoculum was of spleen cells both cytotoxic and plaque cells were produced during the GVH response. However, if thymus cells were the responding cell type only cytotoxic cells were produced. Subsequent studies by Cerottini et al (231,232) demonstrated that elimination of T cells from a mixed population of cytotoxic and plaque-forming cells caused a loss of cytotoxic activity without any concomitant decrease in plaque-forming cells. Although these experiments demonstrated that T cells were necessary for the manifestation of cytotoxic effects in alloimmunised cell populations they did not prove that T cells were the actual effectors. However, Goldstein &

Blomgren (233) showed that as few as 10^3 cells of a highly purified cytotoxic T cell population were able to cause significant lysis of target cells, thus making it extremely unlikely that other cell types are involved. Further evidence supporting this conclusion has recently been discussed (234).

Coupled with studies of the MLC reaction showing that purified populations of virgin T cells can transform into cytotoxic cells (reviewed in refer. 112) there is a strong case to support the notion that cytotoxic T cells are the mediators of allograft destruction in vivo. Even so, direct evidence to support this contention is at the moment lacking. Various workers have established that cytotoxic cells active in the chromium release test are generated in vivo after injection of either allogeneic cells (17,235-237), or following implantation of either allogeneic skin grafts (194,238,239) or kidney grafts (240,241). In most cases cytotoxic activity was detectable 3-5 days after immunisation, reached a peak at 6-10 days, then declined rapidly. In many cases cytotoxic cells were detected first in the draining lymphoid organ and reached highest activity there. Whether these cells are on their own able to cause graft destruction is, however, not clear at the moment. Sprent and Miller (242) showed that cytotoxic T cells recovered from the thoracic duct of irradiated mice in which a GVH reaction had been induced caused rejection of both allogeneic skin grafts and allogeneic tumour inoculi when injected into neonatally thymectomised mice. Yet the same spleen cells did not induce a splenomegaly GVH reaction in neonatal F_1 mice. A similar ability of cytotoxic T cells to prevent tumour growth in lethally irradiated animals was reported by Brunner and coworkers (243), the protective effect of the cytotoxic T cells being abolished by treatment with anti- θ serum. In some of these experiments it was necessary to administer normal spleen or bone-marrow cells to

prevent irradiation death. The assumption that these cells are inert has been put in doubt by experiments of Rouse and Warner (244). These authors reported that cortisone-resistant thymocytes activated in vitro were able to mediate skin graft rejection in thymectomised, irradiated, bone-marrow reconstituted mice but not in lethally irradiated mice. The results suggested that cytotoxic T cells are not sufficient for graft destruction and that either bone-marrow-derived cells or possible other types of T cell are required. They also found that, although their cytotoxic T cells were unable to cause GVH reactions in newborn F_1 mice (in agreement with Sprent and Miller's findings) they could nevertheless proliferate strongly in irradiated adult F_1 mice, indicating that a loss of proliferative ability was not the reason for their impotence in GVH reactions. They postulated that cooperation was required for GVH reactions (144) and that the highly selected killer T cell population lacked a second and vital cell type. Thus, present indications are that unless cytotoxic T cells are intimately mixed with target cells either in vitro (17,230-244) or in vivo (243) they are not by themselves able to cause graft destruction even though they can clearly recirculate (242). This may be because they have a short life span and recruitment of other T cells or other cell types able to cause cytotoxicity is a vital aspect of cell-mediated allograft destruction. Further evidence bearing on this point and the available alternative effector mechanisms will be discussed in the next section.

(iv) Alternative effector mechanisms.

There is considerable evidence that suggests that during both the initiator and effector stages of the allograft response "non-specific" recruitment of cells may be important. Thus, although most of the cells in rejecting skin grafts are the products of recent cell divisions (164), the cells which arrive at the graft are not on the whole

specifically activated against graft alloantigens (245). Indeed experiments on the adoptive transfer of sensitivity have shown that the great majority of cells localising in sites of delayed hypersensitivity reactions (246) or in skin allografts (245,247,248) are derived from the host and only very recently (249), using sensitised cells heavily labelled with I-125 IUdR, has it been possible to demonstrate any specific localisation of sensitised cells in target grafts. That non-specific cell recruitment is induced by a small number of specifically reactive cells has been suggested by experiments in which adoptive transfer of either delayed hypersensitivity (250) or allograft immunity (251) was inhibited by irradiation of the recipient. Similarly, the fact that GVH reactions can also be inhibited by prior irradiation of F_1 recipients (208), coupled with the finding that recipient cells are observed to proliferate both at the reaction site and in the local lymph nodes (207), argues against the explanation that recipient lymphocytes act only by providing antigen. Najarian and Feldman (247,248) found that when sensitised cells were placed in Millipore diffusion chambers in close proximity to skin grafts the grafts suffered accelerated destruction; this is not easily observed when the chambers are implanted at a more distal site (247,252-254). Fujimoto et al (252) also found that normal cells placed in a diffusion chamber with allogeneic epidermal cells could not destroy them, whereas sensitised cells could. Since it is now known that epidermal cells can sensitise allogeneic lymphocytes in vitro (181-185), the original interpretation of these experiments, that cell recruitment was essential for target cell destruction, seems valid.

A rationalisation of these experiments was first provided by the work of Willoughby and colleagues (255). They showed that a soluble material obtained by ultrasonication or freezing and thawing of sensitised or normal lymph node cells caused increased

vascular permeability when injected intradermally into a non-sensitised recipient animal. This factor, named lymph node permeability factor (LNPF), also caused massive leucocyte migration from blood vessels and accumulation of mononuclear cells at the site of injection similar to those found in tuberculin reactions (256). Indeed, a material with identical properties to LNPF could be isolated from tuberculin-induced skin reactions (256).

Subsequent work has shown that antigen-stimulated lymphocytes can release a great range of soluble factors including factors able to inhibit macrophage migration (257), as well as cytotoxic (258), mitogenic (259), and chemotactic (260) factors. Injection of factors from antigen-stimulated cells can cause tuberculin-like reactions in the skin (261) and cause an accumulation of cells in local lymph nodes (262) similar to that seen in delayed hypersensitivity and allograft reactions. Furthermore, it has been reported that migration inhibition factor and mitogenic factor can be detected in the circulation during tuberculin reactions (263,264). Whether a single factor can have more than a single biological activity is at the moment unknown, but present information on the chemical and physical properties of the factors indicates that migration inhibition factor can be distinguished from chemotactic and cytotoxic factors and that mitogenic factor is different from cytotoxic factor, at least in humans (for recent review see 265). That these factors can be produced as a consequence of transplantation immunity has been shown for migration inhibition factor (266,267) and lymphotoxin (268), and Ramseier has shown that a factor released during the early stages of MLC reactions can induce the accumulation of leucocytes at skin sites (269).

Evidence that these factors may be important in transplantation has come from the work of Willoughby's group. They have presented evidence that the cellular infiltrate in both tuberculin reaction

sites (270) and in skin allografts (271) may be largely composed of bone-marrow-derived cells, but that in order for cell infiltration and skin graft rejection to occur an initial recognition of antigen by T cells is required. Thus, in mice which have been sublethally irradiated (but not thymectomised) skin graft rejection could be induced by injection of bone-marrow, but not lymph node cells; injection of both cell types together caused more rapid graft rejection than bone-marrow cells alone (271). Recently, Cohen has presented evidence that, after incubation of thymus or spleen cells on syngeneic or allogeneic fibroblasts in vitro for 16-18 hrs, they can induce GVH reactions in vivo even after they have been given 2000 R of irradiation (272). During such GVH reactions induced by irradiated cells cytotoxic cells are recruited from the host population - no cytotoxic cells are produced if the host animal is irradiated. These experiments are reminiscent of those performed several years earlier by Elkins (208) in which irradiation of F_1 hosts inhibited GVH reactions. Bach and coworkers (151) have presented evidence for cooperation in the induction of cytotoxic cells during MIC reactions, although in these experiments the possibility that the cytotoxic cells are the progeny of initiator cells cannot be excluded (for discussion of these experiments see Chap. 13 Introduction).

Although cell recruitment may occur via non-specific soluble mediators there are several reports suggesting that specific information may be transferred from sensitised cells to virgin cells. The best known phenomenon in this field is the transfer factor effect, in which dialysable extracts of sensitised cells can confer immunity to unprimed persons (for review see 273). Although there is some doubt about the antigenic specificity of the phenomenon (273), the recent development of an in vitro assay for transfer

factor (274) should greatly accelerate the pace of research in this field. For many years there have also been reports that RNA extracts from sensitised cells can transfer immunity. A big problem with this work has been the possibility that the transferred material is merely antigen or antigen fragments associated with RNA. But the recent demonstration that transfer of plasmacytoma RNA to normal mouse lymphocytes induced them to synthesise immunoglobulins with the same idiotypic specificity as the original plasmacytoma immunoglobulin provides very strong evidence that information transfer between lymphocytes can really occur (275). Such experiments fit well with recent evidence indicating that transfer factor may be a conjugate of peptide-RNA (276), the implication being that it is some kind of inducer or de-repressor which acts on the lymphocyte genome. It may be some time before the influence of such specific mediators on allograft responses can be ascertained.

In addition to the production of cytotoxic T cells, other mechanisms by which lymphoid cells can cause target cell destruction have been identified. One of these, antibody-dependent cell-mediated cytotoxicity was first noticed by Möller (277) and extensively studied by Perlmann and colleagues (278) and by MacLennan and colleagues (82). Normal lymphoid populations can kill target cells provided these have been coated with antibody, and although red cells were originally used as the targets, killing of coated allogeneic lymphoid cells in this system has been reported by several groups (279-281). Extremely low concentration of antibody are required to induce lysis of target cells (282), and target cells coated with as few as 100 antibody molecules are susceptible (282). Indeed, high concentrations of antibody are inhibitory (282). However, the class of antibody is important, IgG but not IgM being effective (283, 284). Pretreatment of lymphocytes with anti-target cell antibody is

extremely inefficient at inducing target cell lysis compared with treatment of the target cells (284,285), whereas pretreatment of lymphocytes with antigen-antibody complexes in antibody excess renders them strongly cytotoxic (286). The nature of the effector cell is the subject of some controversy at the moment. T cells have been ruled out by the demonstration that spleen cells from thymectomised-irradiated rats and mice have normal cytotoxic activity (287,288), and electron microscope studies have shown that lymphocyte-like cells, rather than macrophages, are the effectors (289). Treatment of cell suspensions with anti-immunoglobulin sera + complement does inhibit cytotoxicity (288), but since the effector cell acts via its Fc receptor blockage of this with non-specific immune complexes could account for the inhibition with anti-immunoglobulin sera (290). Indeed recent studies have shown that the effector cells are not removed by passage through columns coated with $F(ab')_2$ anti-immunoglobulin antibodies (291) or through nylon wool columns (292), and that they are present in agammaglobulinaemic patients who have no immunoglobulin-bearing (B) cells (292). Thus, the available evidence supports the contention of Greenberg et al (83) that at least in some systems the effector cell is apparently a null cell, lacking the conventional markers of B and T cells.

A third cell-mediated cytotoxicity system has been delineated by the studies of Alexander, Evans, and coworkers. It had been known for some time that macrophages taken from animals infected with micro-organisms frequently killed non-specifically. Yet Granger and Weiser had demonstrated that macrophages obtained from the peritoneal cavities of tumour-bearing mice were specifically cytotoxic to the same tumour cells in vitro (293). Evans and Alexander confirmed this finding (294), and showed that non-immune macrophages, after incubation with immune spleen cells, became activated and were then able

to kill (294,295). They went on to show that immune lymphocytes released a factor that could arm non-immune macrophages (296), and that such armed macrophages would kill non-specifically after interaction with the specific target cells (295,296). The arming factor is released by activated T cells (297) and the possibility that it is immunoglobulin seems ruled out by physical studies which have shown activity in the molecular weight range 50-60,000 (296,297).

The relative importance of the three mechanisms of cell-mediated killing in the allograft reaction remains unclear. Since none of the evidence presented in the previous section can rule out the participation of very small amounts of antibody sufficient to coat target cells for antibody-dependent cell-mediated lysis, this mechanism may play some role in graft destruction. And, since a large portion of the cells infiltrating skin grafts are macrophages (203), these could become armed after release of arming factor from sensitised T cells and act as major agents in precipitating graft rejection. Two recent studies have in fact indicated that several types of effector mechanism may be operative during rejection of allogeneic tumours. Forman and Britton (298) found that during the first 5 days following ip immunisation, the cytotoxic cells recovered from the spleen were not sensitive to anti- θ treatment, could not be removed by carbonyl-iron treatment, and in vitro killing was increased by adding antibody. After 5 days, both anti- θ sensitive and anti- θ resistant killers were present. In a similar study Grant et al (299) found that by 7 days post immunisation spleens contained what appeared to be antibody-secreting cells and cytotoxic T cells. At 21 days cytotoxic T cells had declined and killing was mediated by what seemed to be armed macrophages since before contact with antigen killing was dependent on a radio-sensitive θ positive cell, but after contact with antigen killing was radio-resistant. This activity could well

have been present at 7 days but for technical reasons could not be separated from direct T cell killing.

It thus appears that all three cell-mediated killer systems may be operative either simultaneously or at different stages of the allograft response. The inevitable presence of plasma cells simultaneously with cytotoxic cells (194, 230-232) provides a further complication and it will certainly be many years before the nature of the effector cells in any given type of allograft response can be identified unambiguously.

(v) Situations in which antibody can cause allograft rejection.

There is no doubt that passive transfer of alloantibody can cause accelerated rejection of dissociated lymphoid cells (300,301) even when they are enclosed in a Millipore diffusion chamber (252). However, as already discussed there is much evidence to show that antibody acting alone is not the cause of rejection of solid tissues, and this is supported by the failure of presensitised hosts to cause rejection of allogeneic tissues (302) or non-lymphoid cells (252) placed in diffusion chambers, or of skin grafts separated from their hosts by a Millipore membrane (173).

On the other hand, a number of workers have shown that breakage of tolerance in ducks (303), rats (304), and mice (305) can occur following injection of antibody. Effects of the injected antibody were usually noted immediately, but grafts usually survived for several days or weeks. This long period before rejection is probably caused by the chimaerism of the recipient; only when chimaerism has been broken can the skin grafts be rejected. In all cases, the recipients on re-challenge with a second graft were found to be no longer tolerant. It has been argued (304) that the antibody acts only on the chimaeric lymphoid cells, and when these are removed antigen-reactive cells reappear in the host and cause the rejection of

the grafts. Against this is the finding that damage to the graft is observed within hours of injecting antibody, and that total graft rejection can sometimes occur in a matter of days (303). Furthermore, there are now a number of recent reports showing that in either normal (306,307) or ALS-immunosuppressed animals (308,309) antibody can cause accelerated graft rejection. Many previous attempts to demonstrate this phenomenon had failed. One reason for this is that in order to demonstrate an effect of antibody, it should ideally be injected after vascular connections have been made with the graft (307,309). Furthermore, effects of antibody can usually only be demonstrated where the grafts have a long normal survival time which can be achieved either in tolerant animals (303-305), with grafts containing only a minor histocompatibility difference (307), or in immunosuppressed animals (308,309). A further important point is the nature of the antiserum. Thus sera obtained early after immunisation are usually more effective than sera obtained from late bleeds (306,307), this apparently being due to increased concentrations of IgG_1 non-complement-fixing antibodies in the latter sera (306). Providing sera with high concentrations of complement-fixing IgG_2 (306) or (presumably) IgM antibodies (307) are used accelerated graft rejection can clearly be demonstrated.

Kidney grafts in several species of animal seem to be very sensitive to the presence of pre-formed antibodies. Thus in dogs (310), goats (311), and rabbits (312) but not in rats (313), hyperacute rejection of kidneys almost inevitably occurs if antibodies are present. Unfortunately this is also the case in humans (314). Presumably the high vascularity of kidneys compared to skin grafts makes them particularly susceptible to pre-formed antibodies.

Thus, there seems to be no doubt that under certain conditions antibody can be the prime mediator of graft destruction. In some

cases interaction with antibody-dependent lymphocytic killer cells (see above) may be the mechanism involved since, at least in rodents, the host animal's own complement system may be insufficient to cause rejection in the presence of alloantibody (309,313). Although there is much data arguing against a participation of antibody in the normal primary allograft rejection, the recent discovery of antibody-dependent killer cells which can operate efficiently in the presence of only minute quantities of antibody, coupled with the fact that antibody production almost inevitably accompanies graft breakdown, makes it impossible to rule out the participation of antibody in the normal rejection of allogeneic tissues.

CHAPTER 2. Materials and Methods.

A. Materials.

1. Anaesthetic.

7.5 gms of sodium nembutal powder (sodium pentobarbitone, Abbot Laboratories, England) were dissolved in a total volume of 1 litre made up from 900 mls normal saline, 70 mls distilled water, 20 mls propylene glycol (BDH, Ordinary Reagent), and 10 mls absolute ethyl alcohol (James Burrough Ltd, England). Mice were injected intraperitoneally with 0.01 mls/gm body weight.

2. Citrate.

Tri-sodium citrate (BDH, Analytical Reagent) was dissolved in distilled water to give a 4% w/v solution. It was sterilised by autoclaving.

3. Culture Media.

RPMI 1640 medium was obtained in powder form in "1 litre" packets (Grand Island Biological Company, USA). The contents of either one or two packets were dissolved in an appropriate volume of water (de-ionised, then distilled) together with 2.0 gms/litre sodium bicarbonate (Analytical Reagent Grade) and the antibiotics cloxacillin and ampicillin (Beecham Laboratories, England) at a concentration of 125 microgm/ml. A small residue of material from the RPMI powder never dissolved completely, even after prolonged stirring. The pH was adjusted to pH 7.0 by addition of a few drops of concentrated hydrochloric acid, then the medium sterilised by filtration through either Carlson-Ford Seitz Filters or Sartorius Membrane Sterilisation Filters. In later batches of RPMI medium, HEPES (BDH, England) was added at the same time as the other constituents to a final concentration of 15mM. The pH was adjusted to pH 7.2 with a concentrated solution of sodium hydroxide (BDH,

Analytical Reagent). After filtration medium was stored in 100 ml bottles at 4° for no longer than 5 months.

Eagle's MEM was obtained as a 10X strength solution from Wellcome Laboratories, England. For culture it was diluted with de-ionised-distilled water, antibiotics and bicarbonate being added to the same concentration as for RPMI medium.

4. Ficoll-Hypaque.

6.35 gms Ficoll (Pharmacia, Sweden) and 15 mls of Hypaque (a 66% solution of sodium diatrizoate BP in water) were dissolved in 100 mls of de-ionised-distilled water and autoclaved.

5. Foetal calf serum.

Supplied by Flow Laboratories, Scotland. Each time a new batch was required, samples of several batches were tested for their ability to support mouse MLC reactions. The serum giving an optimal combination of high cell survival, high levels of DNA synthesis, and high index of transformation was chosen for future work. One batch of serum was obtained from Difco Laboratories, USA. All foetal calf serum batches were inactivated by heating at 56°C for 1-1½ hrs and stored at 4°C.

6. Glutaraldehyde.

Obtained as a 25% aqueous solution from TAAB Laboratories, England. It was diluted to 7.5% in normal saline.

7. Hibitane.

0.5% chlorhexidine in 70% methylated spirit, made up in St. Mary's Hospital, London.

8. Liquid Paraffin, BP.

From Boots Pure Drug Company, England.

9. Mouse strains.

The mouse strains used in this study were as follows:

Full Name	H-2 allele	Abbreviation used	Location of parent colony	Year of separation from parent colony
A/Jax	a	A	Porton Down, England	?
Balb/c	d	B	Institute of Child Health, London	1967
CBA/Caf	k	CBA	Harwell, England	1954
C3H/He	k	C3H	NIH, Bethesda, USA	1958
C57B1	b	C57	Harwell, England	1962
DBA/2	d	D	Imperial Cancer Research Fund Laboratories, Mill Hill London	1971

F₁ hybrids between appropriate strains were used for tolerance induction and as donors of stimulator cells for MLC. All mice were maintained on Compound Oxoid Breeding Diet and tap water ad libitum and were housed in cages giving 10-20 square inches per mouse. The temperature of the animal house was kept at close to 70°F.

10. Normal saline.

A 0.85% w/v solution of sodium chloride (Analytical Reagent Grade) in distilled water. Sterilised by autoclaving.

11. Phytohaemagglutinin.

Reagent grade PHA was obtained from Wellcome Laboratories, England, in 5 ml freeze-dried ampoules. It was reconstituted to 5 mls with normal saline.

12. Radioactive materials.

Methyl-H-3 thymidine (TRK.120; Amersham, England) was obtained as a sterile aqueous solution in vials containing 1 mCi in a total volume of 1 ml at 15-18 Ci/mole. This was diluted with cold thymidine solution to give labelling material of the desired specific activity and total thymidine concentration (see Chapter 3, Section 4). 10 or 20 microl portions were added to cultures.

2-C-14 thymidine (CFA-219; Amersham) was supplied as a sterile solution in vials containing 50 microCi in a volume of 1 ml at 62 microCi/mmoles. This was diluted 12.5 times with Eagle's MEM and 20 microl portions used to label cultures.

Sodium Cr-51 chromate (CJS-1P; Amersham) was supplied as a sterile isotonic saline solution at 6 microgrammes sodium chromate/ml, specific activity 1 microCi/ml. It was used within 6 weeks of the reference date.

13. Ringer Phosphate.

This contained the following salts per litre: 7.78 gms NaCl; 0.745 gms KCl; 0.66 gms $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 0.61 gms $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. This was buffered with 1 mM sodium phosphates to give pH 7.6. All reagents used were Analytical Reagent Grade. Solutions were sterilised by autoclaving (phosphate and Ringer solutions autoclaved separately).

14. Solutions for washing discs for scintillation counting.

Trichloroacetic acid (BDH, Ordinary Reagent) was used as a 10% w/v solution in distilled water, kept permanently at 4°C.

Perchloric acid (BDH, Analytical Reagent) was diluted to 3% w/v in distilled water and kept permanently at 4°C.

Ethyl alcohol (RR grade; James Burrough Ltd, England) was used either at room temperature or at 4°C.

Diethyl ether (BDH, Ordinary Reagent) was used at room temperature.

15. Target cells.

The target cells used for cytotoxicity tests were the EL4 C57B1 lymphoma, the P-815-X2 DBA/2 mastocytoma - both these were kindly provided by Dr. A.W. Boylston of the Department of Experimental Pathology, St. Mary's Hospital Medical School, London - and an A strain lymphoma kindly donated by Dr. K.T. Brunner of The Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland.

EL4 cells were grown in vitro in CMH/10%FCS in either small coffin-shaped flasks (Flow Laboratories, Scotland) or 4" Petri dishes (Sterilin, England). The other two tumours were grown in vivo in the appropriate parental strains and were passaged weekly.

16. Thymidine.

Thymidine from Sigma Laboratories UK Ltd. was dissolved in normal saline to give a 1.5×10^{-3} M solution, assuming 90% purity. The solution was sterilised by autoclaving.

17. Trypan Blue.

Dialysed Trypan Blue was kept as a 1% w/v stock solution and diluted 10X in normal saline just before use. For viability tests, cell suspensions were usually diluted 50 or 100 times in the diluted Trypan Blue. After about 2 minutes cells were counted under phase-contrast illumination.

B. Methods.

1. Mixed lymphocyte culture.

A detailed report of an investigation into various parameters of the mouse MLC reaction is given in Chapters 3 and 4. The starting point for these studies was the mouse MLC technique of Festenstein (315-317). With various modifications (see Chapter 3) it proved possible to adapt this technique for use in microtest plates (Chapter 3, Section 6d): these have the advantages of economy of both cells and materials, and much greater ease of handling. The general method for mouse mixed lymphocyte culture in microtest plates which was used for the studies in this thesis is as follows.

(a) Preparation of cell suspensions.

Mice were killed in an atmosphere of either coal gas or of nitrogen containing 8% carbon dioxide. After complete immersion in Hibitane for a few seconds mice were opened up and appropriate lymphoid organs removed in sterile conditions into a small bottle

containing 1-2 mls of neat cooled foetal calf serum or CMH containing 20% FCS. In most cases mesenteric lymph nodes were the source of responder cells, but in experiments where higher numbers of cells were required from each mouse deep and superficial axillary nodes, cervical nodes, and inguinal nodes were also removed. Except in very rare cases spleens were not used as a source of responder cells because of their poor responsiveness in MLC compared to lymph node cells (Chapter 3, Section 9a). Stimulator cells were, in the great majority of experiments, obtained from mesenteric lymph nodes; in some experiments they were obtained from spleens. In all experiments described below the source of the particular responder and stimulator cells used is noted. Because of the effect of donor age on the responsiveness of cells in MLC (Chapter 4, Section 3b) all groups of animals providing responder cells for a given MLC experiment were matched for age and, in the great majority of cases, also for sex.

For preparation of cell suspensions, lymph nodes or spleens were transferred to fine mesh sieves (Young-Archibald & Son Ltd, Scotland), clipped onto 30 ml disposable plastic Universal containers (Sterilin, England). They were teased apart with disposable 23-gauge needles (Gillette, England) and mashed gently with the bottom of a small glass test tube. At frequent intervals the liberated cells were rinsed through the sieve with aliquots of the foetal calf serum (or CMH/20%FCS) into which the organs had been placed originally. 10-20 mls of normal saline containing 2% FCS (saline/2%FCS) were then added to the tubes and the cells spun down at 200g for 10 mins. All ~~supernatant~~ supernatant was removed, the cell pellet dispersed, and a second portion of 10-20 mls of saline/2%FCS added. The cell suspensions were transferred to new tubes and spun as before. Cells were routinely washed 2 or 3 times in saline/2%FCS, then resuspended in

CMH containing 10% (or occasionally 15%) FCS. Where mouse serum was subsequently to be added to cultures, cells were resuspended in CMH containing 20%FCS (see Chapter 4, Section 2). Suspensions were left to stand for 5-10 minutes to allow any aggregated material to settle to the bottom of the tubes, supernatants then being transferred to new tubes.

The concentration of viable cells was assessed by phase-contrast microscopy (Chapter 3, Section 2). The overall viability of cell suspensions prepared in the way described above was 80-90%, and for all in vitro experiments described in this thesis the figures for cell concentration refer to viable cells. Where non-irradiated F_1 hybrid cells were used as stimulators, both responder and stimulator cell suspensions were usually adjusted to 5.0 million per ml - in this way each well in the microtest plate received the optimal dose of 1.0 million cells (see Chapter 3, Section 6e). In a few experiments, especially where mouse serum was used, the cell suspensions were adjusted to 6.0 million per ml. For mixed cultures, equal volumes of responder and stimulator cell suspensions were mixed and 200 microl aliquots of mixed cells, stimulator cells, and responder cells were dispensed with an Eppendorf pipette into the flat-bottomed wells of Falcon 3040 Microtest II plates (Falcon Plastics, USA). Generally 4 replicate wells of each suspension were used, but where higher precision was required this figure was increased to 6 or, very occasionally, to 8. Replicate cultures were usually distributed according to a predetermined random code so as to avoid any systematic variation in culture conditions in different parts of the plate from affecting the outcome of the experiment.

Where irradiated stimulator cells were used, equal volumes of responder cells at 10.0 million per ml (or occasionally at 12.0 million per ml) and irradiated stimulator cells at 5.0 million per

ml were mixed, giving a 2:1 responder:stimulator cell ratio. Control cultures were formed in the same way by mixing responder cells with irradiated syngeneic cells. All irradiated cells received 2000 rads of X-irradiation from a Siemens Stabilipan machine fitted with a 1 mm Cu filter at the rate of 133 rads per minute. This dose of radiation completely abolished the ability of cells to respond in MLC (Chapter 3, Section 7b). Replicates were dispensed into microtest plates as described above.

(b) Culture.

Cultures were incubated according to the method of Festenstein (316,317): they were placed in a desiccator and air was evacuated until the pressure dropped to 400 mm Hg. Gas was introduced from a cylinder containing 92% N₂ and 8% CO₂ (British Oxygen) until the pressure had returned to about 80 mm Hg. less than atmospheric pressure. This procedure produces an atmosphere of about 10% O₂, 4% CO₂, and 86% N₂, the slight negative pressure inside the desiccator helping to maintain the seals. An open Petri dish containing water was kept inside the desiccator to maintain a humid atmosphere. The cultures were incubated at 33-34°C, a temperature which has distinct advantages over 37° for mouse lymphocyte culture (Chapter 3, Section 8).

(c) Labelling and harvesting.

After an appropriate period, usually 72 or 96 hrs, 10 microl of methyl-H-3 thymidine solution containing 1-1.4 microCi was added to each well using a Hamilton repeating dispenser. Cultures were then incubated for a further period of about 16 hrs. The final thymidine concentration in wells was in the plateau region of the dose-response curve for thymidine uptake (see Chapter 3, Section 4).

At the end of the labelling period the cultured cells were re-suspended by pipetting up and down with a 200 microl Eppendorf pipette. (With some batches of FCS it was necessary to remove excess

supernatant counts before resuspending the cells by dumping the medium out of the plate and adding 200 microl of fresh unlabelled medium (see Chapter 3, Section 6A.) One 50 or 100 microl sample was removed from each well onto a small numbered paper disc (1.9 mm diam. 3MM filter paper circles from Whatman, England), each disc being supported on a pin placed in a cork board. Samples were also taken of supernatants or from wells containing medium only in order to measure the retention of labelled thymidine on discs after TCA washing.

When samples had been taken from all wells the remaining 100 or 150 microl of each culture was fixed by adding 1 drop of 7.5% glutaraldehyde solution from a syringe fitted with a 23-gauge needle: this gives a final glutaraldehyde concentration of about 0.5%. If cell counts were not to be performed immediately the microtest plate was stored at 4°C until later in the day or occasionally for 1 or 2 days. No change in cell morphology occurs for at least a week after glutaraldehyde fixation. Cell counts were performed to check that survival of responder cells was satisfactory: survival of CBA lymph node cells after 4 days culture was usually 20-40%.

The samples on discs were allowed to dry for either 2-3 hrs at room temperature or 1 hr in a 60°C oven before they were removed and washed. Two washes, each lasting about 15 minutes with frequent swirling, were performed in 10% TCA solution at 4°C. The discs were then washed briefly with 2 changes of 3% PCA (again at 4°C), 2 changes of absolute ethyl alcohol, and 2 changes of diethyl ether. Discs were spread out on boards, left to dry for about half an hour at room temperature, and then placed in glass scintillation vials. 5 mls of cold scintillation fluid were added to each vial and radioactive counting performed on a Packard Tri-carb liquid scintillation spectrometer. The same gain and channel settings previously determined for

optimal counting of tritium were used in all experiments.

(d) Calculation of results.

Estimates of DNA synthesis in replicate cultures of mammalian lymphocytes are usually distributed log-normally. This was found to be true for the mouse cell cultures described in this thesis. However, because the coefficient of variation between replicate cultures were usually small (mostly less than 10%) their distribution fits closely an arithmetic-normal curve, so that the use of the simple arithmetic mean and standard error is quite permissible and has the advantage over logarithmic methods of ease of calculation and presentation.

In the results described below the values of DNA synthesis are thus given as the arithmetic mean of (usually 4) replicates $\pm$ the standard error of the mean, the values having been rounded off to an appropriate number of significant figures. The stimulation is obtained by subtracting the mean counts obtained in unstimulated control cultures (or in cultures with irradiated syngeneic stimulator cells) from the mean counts in mixed cultures (or in cultures with irradiated allogeneic or semi-allogeneic stimulator cells). The index of transformation is calculated as the quotient of these same quantities. The latter is usually the more meaningful parameter for comparing one set of cultures with another, since it counteracts to some extent variations in the proliferative potential of cells caused by different cell survivals. However, it does have the disadvantage that when the level of background transformation is low small errors in the estimation of this quantity can lead to large errors in the estimation of the index of transformation. Therefore, in most of the MLC results presented below, the background transformation of unstimulated cells, the stimulation obtained in mixed cultures, and the index of transformation are all given.

Significance tests on MLC results were performed by the use of Student's t-test on log-transformed data according to the methods of Snedecor (318). Significance levels are usually indicated by asterisks according to the following convention: * = $0.05 > P > 0.01$; ** = $0.01 > P > 0.001$; *** = $P < 0.001$.

(e) Variants of the MLC technique.

Before and during the adaptation of the mouse MLC technique to microtest plates cultures were performed in 2 ml plastic tubes using a method closely similar to that of Festenstein (315-317). The tube technique differs from that described above for microtest plates in the following ways.

Cell suspensions, prepared as described above, were adjusted to 2.5 million/ml in CM/10%FCS. Washed red cells (or, more exactly, whole blood cells) from the citrated blood of CBA mice were added to both CBA and F_1 stimulator cell suspensions at the rate of 20 microl of packed red cells per 10 ml of lymphocyte suspension. Equal volumes of the two suspensions were then mixed and 0.5 ml aliquots of unmixed and mixed cultures were dispensed into 2 ml plastic tubes (Staines Laboratories, England). Holes were made in the plastic tops of each tube with a hot needle, and tubes incubated in desiccators as described above. After approximately 48 hrs tubes which had not previously been labelled with thymidine were refed by adding a further 0.50 ml of fresh medium, making a total volume of 1.0 ml. This process was repeated at about 90 hrs, first removing 0.50 ml of old medium and adding 0.50 ml of fresh medium.

Cultures were labelled at appropriate times by first reducing the culture volume where necessary to 0.50 ml and then adding 20 microl of radioactive thymidine. After a further 16 hrs of culture the volume in tubes was reduced to about 0.2 ml, the carpet of red cells resuspended, and 2 ml of cold normal saline added. Tubes were

spun at 200g for 10 mins, the volume reduced again to about 0.2 ml, and a second wash with 2 ml of cold saline performed. The volumes were then reduced to 0.50 ml, cells resuspended, and duplicate 100 microl samples removed onto paper discs. These were processed in the manner previously described and the remains of cultures fixed in glutaraldehyde for cell counts.

During the transition period in which the tube method was adapted to microtest plates, the need for red cells, for refeeding, and for washing labelled cells was investigated. Each was found not to be essential (see Chapters 3 and 4), and each was eliminated in turn until the tube technique became identical to that described for microtest plates, except for the different number of cells and medium volume used for tube cultures.

Where MLC results are presented in this thesis the technique used was that described for microtest plates: any deviations from this method, in particular those just described, are given in the footnote to the appropriate Table.

2. Cytotoxicity assays.

(a) With effector cells sensitised in vitro.

In vitro sensitisation was performed using the microplate MLC technique described above, except that when live F_1 cells were used as stimulator cells the responder:stimulator ratio in mixed cultures was 3:1, parallel cultures of responder strain cells alone providing control (non-sensitised) cells for the CML assay. When irradiated allogeneic or semi-allogeneic cells were used as stimulators, control cells were provided by cultures of responder strain cells with irradiated syngeneic cells.

Mixed lymphocyte cultures were harvested after $4\frac{1}{2}$ days incubation by gently resuspending the cells in the microtest wells with a 200 microl Eppendorf pipette and transferring them to Universal tubes.

The cells were spun at 200g for 10 mins and resuspended in fresh CMH containing 20% FCS. Viable cell counts were performed by phase contrast microscopy and the cell concentration adjusted to that desired (see below). The yield of viable cells after $4\frac{1}{2}$ days culture was usually 20-30% in control cultures and somewhat higher in stimulated cultures.

About 2 million tumour cells of the appropriate strain were incubated at 37°C for 1-1 $\frac{1}{2}$ hrs in CMH/20%FCS mixed with 200 microCi of sodium 51-Cr-chromate solution to give a total volume of 1 ml, mixing the cells at 10 minute intervals. The cells were then washed twice with 5 ml of CMH/20%FCS to remove supernatant radioactivity, resuspended in the same medium, and counted. The cell concentration was adjusted to between 5 and 10×10^4 per ml and effector cells from control and antigen stimulated mixed lymphocyte cultures added to give the desired effector:stimulator cell ratio. Triplicate or quadruplicate 200 microl aliquots were dispensed into the wells of a microtest plate and incubated in a gassed desiccator (as described in the MLC technique) at 37°C for an appropriate time.

At the end of the incubation the cells were resuspended with a 200 microl Eppendorf pipette and each culture transferred to 0.5 or 1.0 ml round-bottomed tubes. These were spun down at 200g for 10 mins, then 100 microl aliquots removed into small plastic tubes. Chromium release was measured by counting these in a Packard Auto-Gamma spectrometer.

Maximum chromium release was assayed by freezing and thawing 200 microl mixtures of effector and target cells 3-4 times in a second microtest plate. Samples were prepared for gamma counting as described above for incubated cultures. Usually 80-90% of total chromium was released into the supernatant by this treatment.

Percentage chromium release was calculated according to the

standard equation (17):

$$\% \text{ Cytotoxicity} = \frac{\text{Cr release in the presence of sensitised cells} - \text{Cr release in the presence of control cells}}{\text{Cr release caused by freeze-thawing} - \text{Cr release in the presence of control cells}}$$

Using this technique for the assay of cytotoxic cells the % cytotoxicity increased linearly with the logarithm of the effector:target cell ratio between about 15 and 90% killing (Chapter 3, Section 11).

(b) With effector cells sensitised in vivo.

Cells were obtained from the spleens of either unimmunised mice or mice injected intraperitoneally 5 days previously with semi-allogeneic spleen cells. The assay for cytotoxic activity in such cells was performed in exactly the same way as described for assaying in vitro sensitised cells.

3. Preparation of serum and red cells from mice.

Red cells for haemagglutination tests were obtained by bleeding nembutal-anaesthetised mice from the heart into a syringe containing about 0.1 ml of 4% sodium citrate. They were washed 4 times with normal saline before use.

Red cells for cell culture were obtained after bleeding mice according to the method described by Festenstein (316). Mice were killed by asphyxiation in an atmosphere of 92% N₂ and 8% CO₂, dipped immediately into Hibitane, and the thoracic cage then cut open. While the heart was still beating 0.2 ml of 4% citrate was injected into it and then the heart and major blood vessels cut with scissors. The blood which collected above the diaphragm was removed with a Pasteur pipette to a Universal tube and washed 1-2 times with 20 ml of saline/2%FCS.

Blood for the preparation of serum was usually obtained by the method of Festenstein described above, except that the heart was not first injected with citrate. On a few occasions it was obtained by

bleeding anaesthetised mice from the heart by syringe. The former method of collecting blood for the preparation of serum to be used in MLC has advantages other than convenience (see Chapter 4, Section 2). Blood was allowed to clot at 4°C , then spun at 800g for 15 mins at 4° . Serum was removed and spun again at the same speed to remove any contaminating red cells or debris. The clear supernatant was transferred to a new tube and either used straight away or frozen to -20 or -80°C in sealed ampoules.

4. Preparation of hyperimmune sera.

CBA anti-A hyperimmune serum was prepared by injecting 83 5-month old CBA female mice ip with A strain spleen cells at week 0 ($37\frac{1}{2}$ million), at week 3 (25 million), and at week 7 (5 million). Anaesthetised mice were bled 23 days later by cardiac puncture. Serum was stored in sealed ampoules at -20°C .

A second batch of CBA anti-A hyperimmune serum prepared in a similar way and used in experiment B of Table 34, Chapter 4, was kindly provided by Dr. P.J. Kilshaw of this department.

CBA anti-C57Bl hyperimmune serum used routinely as a positive inhibitory control in appropriate in vitro serum tests was prepared in the following way. 15 4-6-month-old CBA males were injected ip with 25 million C57Bl spleen cells at weeks 0 and 2. At week 5 they received 25 million (CBAXC57Bl) F_1 spleen cells, and at week 7 a further 5 million F_1 cells. 10 days later they were bled by the method of Festenstein (see above) and the resulting serum stored at -80° in sealed ampoules.

A second batch of CBA anti-C57Bl hyperimmune serum and control CBA anti-CBA serum was specially prepared for the experiment shown in Table 35 in Chapter 4. The details of its preparation are given in Chapter 4, Section 4.

5. Haemagglutination tests.

The classical method of Gorer and Mikulska was used (319). The

test was carried out in 1 ml glass tubes (Franco-British, England) washed thoroughly in chromic acid. The antiserum diluent was a 1% solution of dextran (Dextraven 110; Fisons, England) in normal saline. Mouse erythrocytes (whole blood cells) from the citrated blood of A-strain mice were washed 4 times in normal saline and resuspended in a 1:1 mixture of normal saline and human serum; the latter had been absorbed twice with mouse red cells (whole blood cells) in a 1:4 ratio at 37°C for 1 hr.

A 50 microl aliquot of diluent was placed in all tubes and serial dilutions were made from 50 microl of antiserum. 50 microl of red cell suspension was added and the tubes incubated at 37°C for 90 minutes. Agglutination was estimated microscopically, a sample from each tube being taken with a Pasteur pipette and streaked on a microscope slide which was gently rocked to and fro to ensure even dispersion of the cells. Agglutination was measured according to an arbitrary scale, 5+ to 0, the end point of the titration being taken as the last tube showing any sign of agglutination.

6. Preparation of cell suspensions for injecting mice.

Donor mice were killed with coal gas and spleens removed into a sieve about 1½" in diameter. The grid of the sieve was kept immersed in Ringer-phosphate in a 4" Petri dish. The spleens were cut into small pieces with scissors and mashed gently with the plunger of a 5 ml disposable syringe as a pestle. The resulting cell suspension was transferred to a 40 ml glass centrifuge tube or a 30 ml plastic Universal container and gently pipetted up and down for 2-3 minutes to break up tissue fragments. Residual clumps were allowed to settle to the bottom of the tube for 10 minutes, then the supernatant decanted into a new tube and spun at 200g for 10 mins. The cell pellet was resuspended in an appropriate volume of Ringer-phosphate, and a cell count made in 0.1% Trypan Blue: viability was usually in the

range 60-70%. The cell concentration was adjusted to the appropriate level. For in vivo experiments described in this thesis the cell concentrations always refer to total numbers of white cells, live + dead.

Bone-marrow cells were obtained from the femur, tibia, and iliac crest bones by cutting off the ends of each bone and rinsing out the marrow with Ringer-phosphate from a syringe fitted with a 23-gauge disposable needle. Clumps were broken up by sucking suspension back into the syringe and re-expelling it through the same needle.

For induction of tolerance in CBA mice to C57Bl and DBA/2 strain antigens it was expedient to use suspensions with as high a viability as possible, since the number of cells that can be injected into a newborn mouse intravenously is limited to about 20 million. To prepare spleen cell suspensions for this purpose, spleens were cut into 2 pieces and cells gently squeezed out into Ringer-phosphate by stroking with the plunger of a 5 ml disposable syringe. Tissue clumps were dispensed by gentle pipetting with a wide tipped Pasteur pipette. The remaining stages of preparation are as described above. The preparation of bone-marrow cells is by nature a gentle procedure and no special precautions were needed. The viabilities of cells used for iv injection of newborn mice were always close to 80%.

7. Induction of tolerance in newborn mice.

Mice used for tolerance induction were always less than 24 hrs old. Cells were injected from a 0.500 ml glass tuberculin syringe fitted with a 30-gauge needle. It was important to ensure that the needle tip was sharp and that the cell suspension flowed freely through it.

For intraperitoneal injections mice were held in the left hand with their legs between the first and second fingers, their left shoulder resting on the third finger, and the thumb on top of the

mouse's right shoulder. Gentle pressure between the thumb and third fingers restrained all head and body movements. The needle was inserted behind the right ear and was passed between the skin and body wall towards the stomach. The tip was then allowed to penetrate the peritoneal membrane and 0.10 ml of cell suspension injected. The needle was very slowly and carefully withdrawn so as to avoid tearing the peritoneal membrane: slight stretching of the skin with the thumb of the left hand helped to seal the injection channel as the needle was removed. If the injection was successful no, or only very slight, back-leakage of injected material occurred. In about 10% of mice, however, significant back-leakage occurred; these were rejected. By removing only half the litter at a time from the cage, and providing the mothers with ample straw and tissue paper to build a warm nest, maternal cannibalism could be kept to acceptable limits.

For intravenous injection, newborn mice must be held completely immobile and yet sufficiently gently to allow free blood-flow and respiration. The author is indebted to the assistance of Miss Anne Thomas for her skillful holding of the mice during this operation. Up to 0.075 ml of cell suspension can then be injected into one of the blood vessels of the sigmoid sinus, entering just above the ear. Careful observation of the blood vessel was maintained during the injection to ensure that all the injected material was passing through it. If the needle came out of the blood vessel injection could sometimes be continued on the other side of the head. Where injections were not successful mice were rejected. For further details of the technique of intravenous injection of newborn mice see references 320 and 321.

8. Induction of unresponsiveness in adult mice.

Mice were made specifically unresponsive to allogeneic skin grafts by the method of Pinto et al (322). Male CBA mice, approx-

imately 3 months old, were injected iv with a crude extract of allogeneic liver prepared according to the method of Medawar (323), each mouse receiving extract derived from 250 mgms wet weight of liver. 14 days later they received an iv injection of *Bordetella pertussis* vaccine (Burroughs Wellcome and Co, England) containing 12×10^9 dead bacteria in about 0.3 ml, and after a further 2 days received a graft of tail skin from the appropriate allogeneic strain. 0.5 ml of rabbit anti-mouse lymphocyte serum, prepared as described by Levey and Medawar (324), was administered ip 2, 4, and 6 days after grafting. 30-50% of mice so treated retain their allogeneic skin grafts in perfect condition for at least 100 days (322).

9. Skin grafting.

Anaesthetised mice were grafted with allogeneic tail skin using the method of Billingham and Medawar (325). Apart from mice in which unresponsiveness was induced in adult life, skin grafts were usually applied when 5-7 weeks old. Plaster dressings were removed 10 or 14 days after grafting, the later time of removal being reserved for mice given a tolerogenic treatment at birth. Graft survival was assessed by visual examination, the percentage of viable epidermis being estimated to the nearest of the following scores: 100% (perfect) 100% (slight blemish), 90%, 75%, 50%, 25%, 10%, and 0. Only tolerant and unresponsive mice having grafts in the first of these categories were accepted for the in vitro tests described in this thesis.

Three problems are encountered with this method of measuring graft survival. One problem is that some grafts are damaged by non-immunological processes, such as graft slippage, infection, and scratching and chewing by recipient mice. In mice given a tolerogenic dose of cells at birth, such damage is clearly distinguishable from immunological damage since these grafts subsequently recover and after 2-3 weeks are in perfect condition apart from a small bald patch

or scar. Furthermore, as described in Chapter 5, Section 2, when such mice are regrafted on the opposite flank the second grafts are in the great majority of cases retained in perfect condition indefinitely. In mice which are not tolerant such non-immunological damage can be mistaken for rejection, but since (as judged from its incidence in tolerant mice) it is relatively rare, affecting only about 10% of graft recipients, it does ~~not~~ have a significant effect on the results of experiments.

A second problem is that a number of grafts show a sudden breakdown in which large portions or even the whole graft becomes covered with a serous-like secretion within 24 hrs of unbandaging. This strange form of graft rejection only began to occur after the mouse colony was moved from Southampton to its present location at St. Mary's Hospital Medical School, London. A similar phenomenon has been reported by Anderson and Cooper (326) to occur in mice at the National Institute of Medical Research, London. Although an environmental factor presumably plays some role in this atypical rejection process, its origin is almost certainly immunological since it is never observed in mice made tolerant at birth and seems to occur less frequently in mice given ALS treatment than in normal mice (326). Histological examination of lesions revealed a dense polymorphonuclear infiltrate under a thick keratinised layer (327). In contrast to the damage caused by non-immunological processes, it can sometimes affect a large percentage of mice in a given experiment, being especially common in CBA mice given C3H/He grafts. In such cases the whole experiment has to be abandoned since estimation of an MST is impossible. More commonly, though, only a small percentage of mice show this atypical breakdown pattern, and by eliminating such mice from the assessment of the experiment a meaningful estimate of MST can be obtained without introducing any significant bias into the experiment.

The third problem is that in a significant proportion of unresponsive and partially tolerant mice graft breakdown occurs over a very extended period, sometimes lasting 2-3 months (see Chapter 5, Section 2). This chronic rejection is characterised by a gradual loss of hair and by graft contraction, and determination of even an approximate end-point of graft rejection is very difficult. Furthermore, since many grafts linger on in a partially rejected form for many months, the usual method of expressing skin graft survivals, where the percentage of mice showing any graft survival is plotted against time, under-estimates the degree of rejection that has occurred. To overcome these two problems, each graft was scored according to the approximate percentage of healthy epithelium, as described at the beginning of this section. The mean percentage survival was then calculated for the whole group and plotted against time. The median survival time is then defined as the time when the mean percentage survival has fallen to 50%.

CHAPTER 3. Preliminary studies of MLC technique.

Introduction.

Following the initial observation of Schrek and Donelly (328) that leucocytes from different individuals underwent blast transformation when mixed together in culture, Bain et al (15) and Bach and Hirschhorn (16) provided the first detailed studies of the mixed lymphocyte reaction (MLR). They showed that when blood leucocytes from unrelated persons were cultured together a proliferative response occurred which was not observed when the cells were obtained from identical twins. These genetic requirements indicated that the in vitro response was analogous to the allograft reaction observed in vivo and although intense study of the MLR over the last 10 years has shown that the correspondence between in vivo and in vitro reactions is not perfect, there are still good reasons for believing that the MLC reaction reflects to some extent the events occurring during the initiation phase of anti-allograft responses. The technology of MLC has recently been admirably reviewed by Sorensen (329) and therefore the introduction to this chapter will emphasise some of the more recent developments in the study of mixed lymphocyte reactions.

(i) The responder cell in MLR.

Clancy and Rieke (330) demonstrated that thoracic duct cells gave strong reactions in MLC, thereby implying that the small lymphocyte which had been shown to respond in GVH reactions (197) was also the responder cell in MLC. That the responder cell was almost certainly a T cell was shown by the finding that lymphocytes from neonatally thymectomised rats (331) and mice (332) were poorly reactive in MLC, while those from bursectomised chickens reacted essentially normally (333). Direct confirmation that at least the majority of responder cells were thymus-derived came from studies where irradiated animals

were reconstituted with chromosomally marked thymus and bone-marrow cells (334,335). Similarly, Mosier and Cantor (336) demonstrated that treatment of mouse spleen cells with anti- θ serum almost completely abolished their ability to respond in MLC.

An alternative approach was the analysis of surface markers on normal cell populations after stimulation in MLC. Both Vischer (337) and Jones (338) found that the great majority of blast cells in mouse mixed lymphocyte cultures possessed θ antigen. Vischer found some evidence of an increase in B cell blasts during stimulation, but this was slight. The preponderance of θ -bearing blasts in mouse MLC cultures was confirmed by Hayry et al (339). Using a slightly different approach Andersson et al (340) mixed chromosomally marked CBA T and B cells and found that 89-97% of mitoses in MLC between days 4 and 9 were in the T cell population. When allowance is made for possibilities of stimulation by serum in culture these results present very strong evidence that T cells can, at least under certain culture conditions, be the almost exclusive responder cells in MLC.

However, not all workers have found the same. Using irradiated mice reconstituted with chromosomally marked cells, Piquet and Vassalli (341) reported that the majority of mitoses in their MLR cultures at 3 days were of T cell type, but that by day 4 and 5 the majority were B cell mitoses. Their results are in direct conflict with those of Andersson et al (340), and, since the majority of late mitoses in their PHA cultures were also of B cell type, are similar to those of Geha et al (98) who found that human B cells could enter into a PHA response providing small numbers of T cells were added.

Wagner has reported (342) that neonatally thymectomised mice and adult thymectomised, irradiated, bone-marrow reconstituted mice have spleen cells able to respond in MLC. That their result is at least in part due to the presence of small numbers of T cells in such mice (76)

is shown by the studies of Moorhead et al (343) who found that the MLC responsiveness of spleen cells from adult thymectomised, irradiated, bone-marrow reconstituted mice could be greatly reduced by treating them with anti- θ serum.

Even more remarkable are the findings that cells from congenitally athymic (nude) mice can show some responsiveness in MLC (342, 344). Although it is now known that nude mice have small numbers of θ -positive cells (345), the responsiveness of nude mouse cells in MLC cannot be completely accounted for by the presence of these cells. Croy and Osoba (344) made the important observation that whereas cells from nude mice would respond to irradiated normal allogeneic stimulator cells, they would not respond to T-cell-depleted irradiated stimulator cells. They concluded that the presence of T cells in either the responder or stimulator populations was sufficient to allow an MLC response to occur and that a radiation-resistant recognition process by T cells could induce the non-specific proliferation of B cells.

Strong evidence to support this conclusion has recently been reported by three groups of workers. Kennedy and Ekpaha-Mensach (346) found that the plaque forming ability of F_1 mouse spleen cells cultured with sheep red blood cells was increased by adding irradiated or mitomycin-treated parental strain cells. They went on to show that this could still occur when the MLC mixture was separated by a nucleopore membrane from the mixture of normal spleen cells and SRBC, and also when the normal spleen cells in the SRBC compartment were replaced with cells from B mice. The generation of such B cell helper factors, apparently similar to those found to replace the need for T cells in in vitro cooperation experiments (128-130), has been confirmed by von Boehmer (347). He found that, whereas electrophoretically separated mitomycin-treated B cells could not stimulate allogeneic nude spleen cells in MLC, normal mitomycin-treated spleen cells were able to do so. Furthermore,

whereas mixtures of (CBA x C57) F_1 spleen cells and mitomycin-treated CBA B cells led to no MLC response, good responses were obtained when F_1 cells were cultured with mitomycin-treated normal CBA cells. Finally, Hirschberg et al (348) found that mixtures of allogeneic mitomycin-blocked human leucocytes which showed no MLC response as measured by DNA synthesis nevertheless secreted into the medium factors able to induce proliferation of normal lymphocytes.

These experiments collectively demonstrate that recognition of allo-antigens by T cells can result in the secretion of proliferation-inducing factors and, most importantly, that the production of these factors, which seem to act primarily on B cells, does not require the synthesis of DNA. Under normal culture conditions such factors play a relatively minor role in MLC responses (334-340), but they produce significant effects in situations where T cell proliferation is limited or prevented, and possibly under certain culture conditions (341). The existence of these factors also explains the previously rather puzzling reports of F_1 cells responding to blocked parental strain cells in MLC (349-351).

(ii) Stimulator cells.

An initial report by MacLaurin (352) that T cells were the major MLC stimulating cells in lymphoid populations of rats has not been confirmed in mice. Thus, Cheers and Sprent (353) found that mitomycin-treated thoracic duct cells from nude mice, which contain almost 100% B cells, gave a similar degree of stimulation to C57Bl spleen cells as did the corresponding cells from nu/+ heterozygous littermates which contain about 80% T cells. This finding that B and T cells have similar MLC-stimulatory powers when there is H-2 incompatibility has been confirmed in a later study (354) in which electrophoretically separated B and T cells were used. Remarkably, however, in strain combinations where there was only an M-locus difference, responses occurred only against fractions containing B cells.

Somewhat different results were obtained by Plate and McKenzie, who found that after treatment of mouse lymphocytes with anti- θ serum there was no reduction in their ability to stimulate cells from an H-2 congenic strain in MLC, whereas treatment with an anti-B cell serum caused marked reduction (355). Moorhead et al (343) also reported that anti- θ treatment of normal spleen cells had no effect, but similar treatment of spleen cells from thymectomised, irradiated, bone-marrow reconstituted mice halved their stimulatory power. It thus appears that while B cells are in general strong stimulators in MLC only a subclass of T cells may be able to stimulate. The relatively high stimulation obtained with purified T cells by Sprent and colleagues (353,354) may have been due to contamination of their T cell populations with B cells. Nevertheless, their results do suggest that M-locus antigens are distributed differently among lymphoid cells than the H-2 LD antigens.

Whereas B lymphocytes, at least some T cells, and also macrophages (356,357) are able to stimulate allogeneic lymphocytes in vitro, studies of non-lymphoid cells have shown them on the whole to be non-stimulatory. Hardy and Ling (358) reported that HeLa cells were unable to stimulate lymphocytes from normal individuals, and in a more extensive study of various lymphoid and non-lymphoid cell lines Han (359) found that none of the latter were stimulatory in MLC. Similarly, in the rat, neither kidney (181,182), thyroid (182), or brain (182) cells stimulated in vitro. The one exception seems to be epidermal cells, which have been found to stimulate in rat (181,182), mouse (183) and human (184,185) MLR. Syngeneic epidermal cells do not stimulate, but evidence has been presented that (C57 x A) F_1 cells, after passage in an irradiated C57 host, can respond to A strain epidermal cells, the suggestion being that the tolerance in the F_1 cells for epidermal antigens was lost during the passage (183). In man, whereas epidermal cells from unrelated persons were stimulatory, cells prepared from

HL-A identical siblings did not respond to each other's epidermal cells (184), indicating that epidermal MLC antigens are closely linked to HL-A and may be identical to LD antigens on lymphocytes. However, evidence against this latter conclusion has been provided by Hirschberg et al (185), who used a thymidine suicide technique to eliminate those MLC-reactive cells from leucocytes of person A that could respond to leucocytes of person B and found that the remaining population could still respond to epidermal cells from person B. Since skin cells seem to possess differentiation antigens (213), the possibility that they have MLC antigens distinct from those on leucocytes is not without precedent.

Finally, there are reports that at least in the rat (360) and mouse (361) allogeneic embryonic fibroblasts can stimulate the production of low levels of cytotoxic cells in lymphocyte populations, but in both mouse (361) and man (359,362) fibroblasts induce only minimal or no DNA synthesis in the responder population. In view of the reports that cellular proliferation is a necessary prerequisite for the generation of cytotoxic cells (see below), a more detailed investigation of the fibroblast monolayer system is needed to clarify whether DNA synthesis is a universal requirement for effector cell production.

(iii) Genetics of MLC reactions.

Because MLC stimulation was usually correlated with disparity at the major histocompatibility locus it was usually assumed that stimulation was caused by the serologically-defined antigens. However, two attempts to correlate the degree of stimulation in mouse MLCs with the known serological specificities proved unsuccessful (349,363), and although a third investigation of this point appeared to show some correlation (364) the availability of congenic mice recombinant at the H-2 locus permitted a more exacting genetic analysis. Initial results suggested that the K end of H-2 was responsible for MLC

stimulation (365), but closer investigation by Bach and colleagues revealed that the major part of the stimulation by K end differences was due to the inclusion of Ir region differences (366). When MLCs were set up between cells from recombinant mice differing at selected subregions of the H-2 complex the greatest stimulations occurred when there was a difference in the Ir region; when there was no disparity at Ir, MLC responses were minimal (366,367). The MLC stimulating antigens, which were not serologically detectable, were named LD (lymphocyte defined), and the K and D sub-locus antigens, which were not strongly stimulatory in MLC, but which induced antibody formation, were termed SD (serologically defined).

The results obtained by Bach's group have been confirmed in essence by two other laboratories (368,369), and they correlate well with the earlier studies by Eijssvoegel and colleagues in recombinant human families (370) where MLC stimulation was clearly not caused by differences at either of the two serologically defined sub-loci, and was apparently due to an MLC locus lying outside the second HL-A sub-locus. In both humans (371) and mice (369) there is some evidence for a second minor MLC locus, but because of the lack of markers within the major histocompatibility complexes of man and mouse it is not possible to locate the LD regions exactly. Indeed, it may be that all subregions of the H-2 complex have some stimulatory power, the relative amounts depending to some extent on the culture conditions used (351). It must also be pointed out that, although SD differences are weak stimulators in MLC, the possibility that these antigens can stimulate to some degree has not been eliminated, and the recent demonstration that differences in the Ir region can induce antibody formation (372,373) suggests that the distinction between LD and SD determinants may not be as strong as it was once thought to be. At the moment it is not possible to say whether the LD antigens

stimulating antibody formation are the same antigens that stimulate in MLC.

Some insight into the possible biological functions of LD and SD antigens was provided by the elegant experiments of Eijssvoogel et al (374), which showed that, whereas LD antigens caused strong MLC stimulation, the cells produced were unable to kill PHA blasts in the CML assay (339). However, when the stimulating cells possessed both LD and SD antigens, CML lysis of PHA blasts occurred provided these possessed the same SD antigens as the stimulator population in MLC. Exactly analogous results have been obtained in the mouse (375, 368). It is not possible at the moment to say whether the SD antigens themselves can act as the targets for cytotoxic cells for they may merely be acting as markers for closely linked, non-serologically active, target antigens. Certainly the K and D SD antigens cannot represent the sole targets for cytotoxic cells, because CML activity can be observed against cells of an H-2 mutant, E(z1), which has no serologically detectable differences from C57 strain cells, but which stimulate the latter in MLC and induce the formation of cytotoxic cells (376).

It has been postulated (367) that, because it is not possible to detect cytotoxic cells when there are only LD differences, there is a separate subclass of cells which mediate cytotoxic reactions and that activation of these cells will only occur in the presence of cells responding to an LD stimulus. In support of this Bach et al (151) have shown that pre-adsorption of lymphocytes onto allogeneic adherent cells did not inhibit their subsequent MLC responsiveness to stimulator cells from the same person who donated the adherent cells but caused reduced production of cytotoxic cells. When adsorption was done after MLC sensitisation less than 10% of cells containing newly synthesised DNA stuck, whereas the majority of cytotoxic cells were removed. Although this experiment is consistent with

a two-cell theory, all it really demonstrates is that the adherent cells either lack LD antigens or that cells bearing anti-LD receptors combine with the specific LD antigen with only low affinity. As pointed out by Howard (377), the reported absence of cytotoxic cells in populations stimulated only by LD differences may be because the PHA-blast target cells do not have LD antigens, a possibility supported by reports that T cells have much lower quantities of LD antigens than B cells (see above). Furthermore, Klein has recently reported that some CML activity can be induced in combinations differing only in the Ir region (378). Therefore, although recruitment and cooperation may be important for the generation of cytotoxic cells, the in vitro data is not at the moment sufficiently strong to merit abandoning the more traditional concept that cytotoxic cells are the progeny of initiator (MLC) cells (379).

Whereas in the rat (380) and in humans (381) MLC stimulation is caused only by disparity at the major histocompatibility complex, this is not the case in mice. The extensive studies of Festenstein and colleagues have shown that a second locus, the M locus, causes MLC stimulation comparable or even greater than that caused by H-2 differences (382). This locus is at present not serologically detectable and is not linked to any other known histocompatibility locus in the mouse. Although it has not been separated into a congenic background, available data suggest that it does not influence graft rejection and, interestingly, that although it can cause GVH reactions when assayed by the popliteal lymph node assay, it does not induce splenomegaly unless the host has been preimmunised (383). Furthermore, while MLC stimulation by M locus differences does not induce cytotoxic cells when PHA blasts are used as the test targets, cytotoxic cells are detectable when DBA/2 mastocytoma cells are the targets (368). As it appears that T cells do not possess M locus

antigens (354) the absence of cytotoxicity against PHA blasts is understandable. The results obtained in the M locus studies further emphasise the need for caution in interpreting the results obtained with LD differences at the H-2 locus.

1. Basic technique.

The basic technique of mouse mixed lymphocyte culture on which the studies in this thesis are based is that of Festenstein et al (315-317). During preliminary studies described in this chapter the technique was adapted for use in microtest plates, and various aspects of MLC reactions were studied so as to ensure that an accurate assessment of cellular reactivity in mice could be obtained.

2. Estimating the viability of cultured cells.

The dye exclusion test provides a simple and objective method of assessing cell viability, but the need to make dilutions of cells in the test fluid is time-consuming when many suspensions have to be counted and there have been reports that serum in the medium can interfere with the test (see 384). Therefore the possibility of using phase contrast microscopy to estimate cell viability was investigated, since no prior dilution of cell suspensions is necessary with this technique. Furthermore, this test is applicable to cells fixed with glutaraldehyde (385). The phase contrast method has, nevertheless, the disadvantage of being somewhat subjective and so the following experiment was performed to compare the results obtained by phase contrast and dye exclusion methods.

Cells obtained from the lymph nodes of one C3H mouse were washed once in Ringer-phosphate. 0.1 ml aliquots of this suspension were added to 0.4 ml aliquots of either 0.1% Trypan Blue (freshly diluted with saline from a 1% stock solution) or saline, and cell counts were done 2 minutes after each dilution. The mean viability obtained from 3 assessments by dye exclusion was $64 \pm 7\%$, and from 4 assessments by phase contrast $66 \pm 3\%$. Although the experiment is not free from possible subjective errors, it is clear that there is no important quantitative difference between the two methods.

TABLE 1. The survival of mouse lymphocytes in
various media at room temperature.

Medium	Time	
	0 hrs	3 hrs
Normal saline (NS)	91 $\pm$ 2%	74 $\pm$ 3%
NS + 2% FCS	90 $\pm$ 1%	87 $\pm$ 2%
PBS + 2% FCS	90 $\pm$ 1%	86 $\pm$ 1%
RPMI	93 $\pm$ 1%	92 $\pm$ 1%
RPMI + 10% FCS	92 $\pm$ 1%	90 $\pm$ 1%

3. Preparation of cell suspensions.

In two experiments lymph node suspensions were prepared in Eagle's MEM lacking FCS, the cells being resuspended in medium containing 10% FCS only after the third wash. In both experiments cell survival and DNA synthesis after 4 days culture were extremely low. The implication was that preparation of cells in the absence of serum had irreversibly damaged them. To investigate this effect in more detail the following experiments were done.

(i) The survival of cells kept in various media at room temperature was studied. The lymph nodes and thymuses from 2 CBA female mice were teased apart in FCS and washed 4 times in PBS containing 2% FCS. 5 x 3.0 ml aliquots of washed cells were transferred to separate tubes, and the cells spun down. All supernatant was removed and cells resuspended in 8.20 ml of one of the 5 media to be tested for their ability to maintain cell survival. Four 0.50 ml replicate samples were removed from each tube at time 0, fixed with glutaraldehyde (0.5%) and cooled in an ice bath. 3 hrs later a further 4 replicate samples were taken from each tube and stored in the same way. All samples were then coded, counted by phase contrast microscopy at random, then decoded. The mean viabilities obtained together with standard errors are shown in Table 1.

Only when mouse cells were kept in the complete absence of nutrients (saline) was there a significant deterioration in morphology over a 3 hr period. Cells kept in the absence of serum but in a nutritious medium (RPMI) showed no detectable drop in viability, and the damage occurring to cells kept in saline could be prevented if as little as 2% FCS was added.

The results suggest that if mouse cell suspensions are prepared in the presence of serum, they can subsequently be kept in a serum free medium so long as this provides basic nutrients or,

TABLE 2. The ability of mouse lymphocytes to respond to PHA after washing them in various media prior to culture.

Wash medium	Time of labelling			
	Day 1-2	Day 2-3	Day 3-4	Day 4-5
PBS (no serum) -PHA	60 $\pm$ 10	ND	50 $\pm$ 10	20 $\pm$ 5
+PHA	640 $\pm$ 20		13,800 $\pm$ 300	7,700 $\pm$ 900
Eagle's medium (no serum) -PHA	80 $\pm$ 5	120 $\pm$ 20	100 $\pm$ 20	80 $\pm$ 30
+PHA	1,770 $\pm$ 80	21,000 $\pm$ 2,200	22,100 $\pm$ 1,900	11,500 $\pm$ 800
PBS + 1% FCS -PHA	90 $\pm$ 10	110 $\pm$ 20	190 $\pm$ 30	60 $\pm$ 20
+PHA	1,580 $\pm$ 40	21,300 $\pm$ 1,100	27,600 $\pm$ 1,600	12,700 $\pm$ 1,100
PBS + 4% FCS -PHA	130 $\pm$ 20	180 $\pm$ 10	210 $\pm$ 60	70 $\pm$ 40
+PHA	1,900 $\pm$ 110	25,000 $\pm$ 1,800	26,700 $\pm$ 1,500	13,500 $\pm$ 900

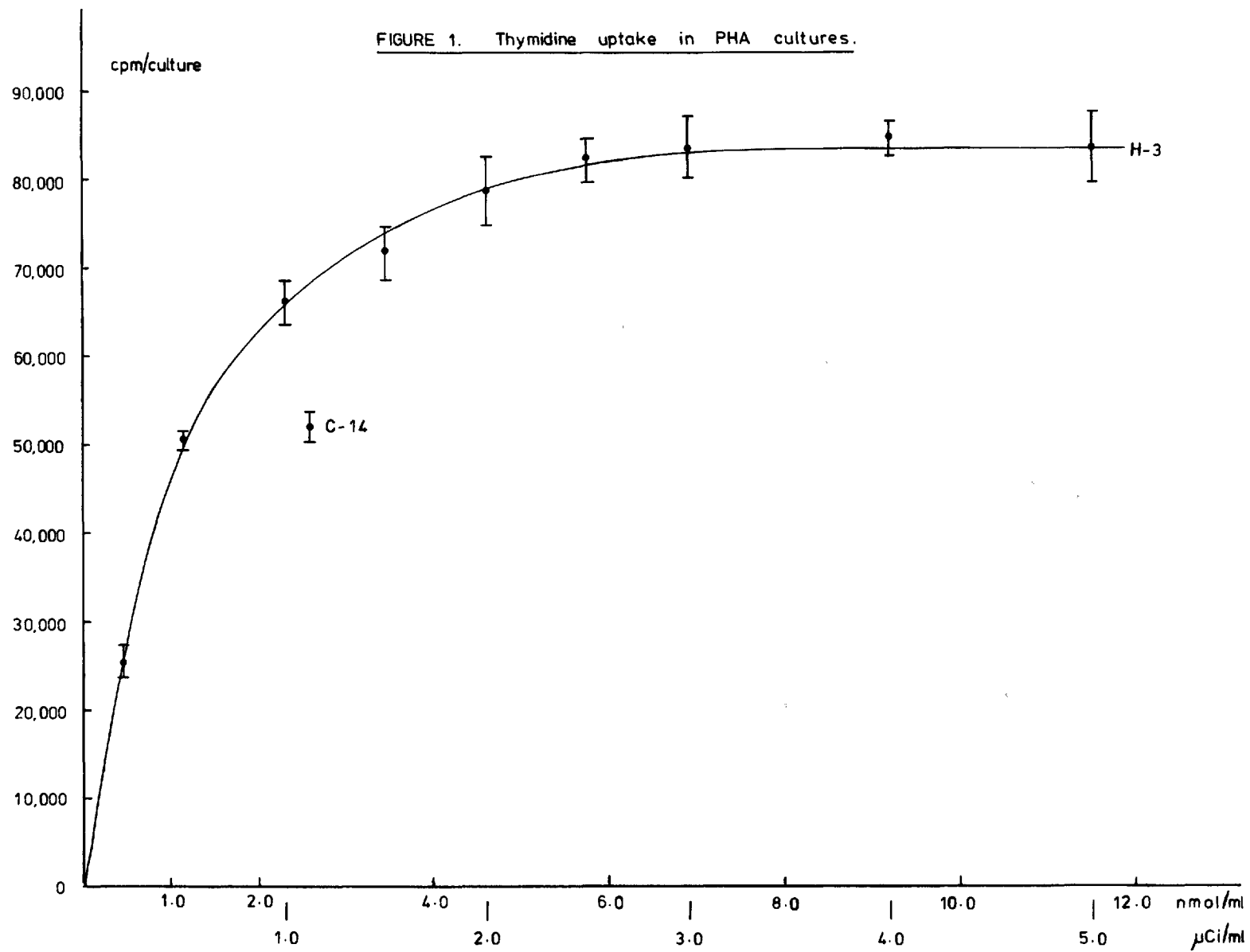
All figures for DNA synthesis are cp5m/100 microl of culture.

alternatively, they can be kept in normal saline supplemented with a small amount of serum. Because morphological examination only provides a crude assessment of cell viability, it was decided to test this conclusion by doing a second experiment where cells would be subsequently incubated with PHA to test their functional viability.

(ii) Lymph nodes from CBA mice were teased apart in foetal calf serum and the resulting suspension of cells in serum divided into 4 approximately equal parts. Cells were then washed 3 times with 4 different wash media, and resuspended in RPMI containing 10% FCS. Concentrations were adjusted to 2.0×10^6 viable cells/ml, the viability of all suspensions at this stage being $85 \pm 3\%$. An equal volume of CBA red cells was added to each, followed by the addition of PHA to portions of these suspensions to give a final concentration of 1/210 in each case. Replicate tubes of control and PHA-containing cultures were labelled after 21, 45, 69 and 90 hrs with C-14 thymidine for 16 hrs. Tubes cultured for longer than 2 days were refed after 48 hrs with 0.5 ml of fresh medium. Results are shown in Table 2.

Cells washed in medium lacking any nutrients (PBS) showed impaired ability to respond to PHA during subsequent culture compared with cells washed in either serum-supplemented medium (PBS + as little as 1% FCS) or serum-free medium containing amino-acids, etc. (Eagle's). This deterioration in the absence of nutrients occurs rapidly for during the first wash FCS was present in the PBS (from the initial cell suspension made in FCS), so that the cells were only in a nutrient-free medium for the last two washes which took about 50 minutes to perform. The results also imply that the failure of the experiments where cell suspensions were made in, and subsequently washed in, Eagle's medium lacking FCS was primarily due to there being no serum present when the cells were released from the lymph node tissue, and not to the washing of the cells in Eagle's medium.

FIGURE 1. Thymidine uptake in PHA cultures.



As a result of these experiments, cell suspensions were always prepared in the presence of FCS (usually teasing tissues apart in neat serum) and routinely washed 2 or 3 times in saline containing 2% FCS.

4. Thymidine labelling.

To ascertain the appropriate concentration of thymidine for labelling cultures two experiments were done.

In the first, varying amounts of H-3 thymidine were added to mouse lymph node cell cultures which had been stimulated in tubes for 3 days with PHA at a final concentration of 1/150. Cultures had been refed with 0.5 ml of fresh medium after 2 days. Four other cultures were labelled with C-14 thymidine. 16 hrs after adding thymidine cultures were harvested. The results are shown in Figure 1.

The uptake of thymidine in PHA cultures plateaued at a concentration of about 6 ~~amoles~~ nmoles/ml. A further doubling of the amount of thymidine added gave no change in the amount incorporated into DNA. If the increasing amounts of radioactive material added had been causing radio-killing of cells a decreased DNA synthesis would have been expected. Thus the plateau of precursor uptake seen in this experiment is due to a saturation of the culture system with thymidine.

In this experiment a comparison was also made of C-14 and H-3 isotopes. A concentration of 2.58 nmol/ml H-3 thymidine (specific activity 0.43 Ci/nmole) gave about 67,500 cpm in the DNA of PHA-stimulated cells. At the same concentration of thymidine but using a C-14 labelled tracer (0.062 Ci/nmole) similar cultures incorporated 52,000 cpm. Therefore, the relative counting efficiency of the two isotopes under these conditions is:

$$\frac{E_{C-14}}{E_{H-3}} = \frac{0.43 \times 52,000}{0.062 \times 67,500} = 5.3.$$

TABLE 3. Labelling kinetics of mixed lymphocyte cultures in microtest plates.

Thymidine solution	Specific activity (Ci/mmol)	Final isotope concentration in cultures (microCi/ ml)	Final thymidine concentration in cultures (nmol/ml)	DNA synthesis in cultures (counts/culture)*		
				Mean of unmixed cultures	Mixed cultures	Stimulation
A	0.25	1.0	4.0	3,220 $\pm$ 100	6,200 $\pm$ 100	2,980 $\pm$ 140
B	0.131	1.0	7.5	3,800 $\pm$ 160	8,000 $\pm$ 100	4,200 $\pm$ 200
C	0.050	1.0	20.0	4,960 $\pm$ 240	9,240 $\pm$ 300	4,280 $\pm$ 400

* Corrected for the different specific activities of the labelling solutions.

Thus, to measure the same number of cpm after incorporation of precursor into DNA it is necessary to use H-3 thymidine at about 5 times the specific activity as would be necessary for C-14 thymidine. However, C-14 thymidine costs 25 times as much per microcurie as does H-3 thymidine, so that it is 5 times cheaper to use H-3 thymidine than C-14 thymidine as label.

In a second experiment the labelling kinetics of mixed lymphocyte cultures in microtest plates were investigated. Lymph node cells from CBA and (CBA x A) F_1 mice were cultured either on their own or mixed in a 1:1 ratio in the wells of a microtest plate. Cultures were incubated at 33-34° and labelled after 78 hrs for a further 16 hrs with 10 microlitres of 3 different H-3 thymidine solutions, A, B and C. Table 3 shows the cpm incorporated into DNA using these 3 solutions after correction for the different specific activities of the solutions.

Although control unmixed cultures continued to show increased thymidine incorporation beyond a thymidine concentration of 7.5 nmoles/ml, those cells responding to alloantigens in mixed culture did not, as shown by there being no significant increase in thymidine incorporation as the concentration of this precursor was increased from 7.5 to 20 nmoles/ml. This may suggest that the synthesis of DNA in cells responding in MLC involves enzymes with a higher affinity for thymidine than those acting in cells responsible for background transformation. The plateau formation cannot be due to radio-killing of transformed cells because in this experiment all cultures received the same total amount of radioactive material.

The kinetics of the two systems investigated here (PHA response in tubes, MLC response in microtest plates) thus appear to be quite similar, requiring a thymidine concentration of 6-8 nmol/ml for optimal labelling. Consequently, in the great majority of experiments

TABLE 4. Comparison of two media for MLC.

		Mean background ⁺ in unmixed cultures	Stimulation in ⁺ mixed cultures	Index ⁺⁺	Survival of CBA cell (%)
<u>Day 2-3</u>	Eagle's	1,220 $\pm$ 45	560 $\pm$ 65	1.46 $\pm$ 0.06	33 $\pm$ 5
	1:1	1,520 $\pm$ 40	940 $\pm$ 190	1.62 $\pm$ 0.13	30 $\pm$ 1
	RPMI	1,380 $\pm$ 65	1,100 $\pm$ 190	1.80 $\pm$ 0.16*	30 $\pm$ 2
<u>Day 3-4^a</u>	Eagle's	1,720 $\pm$ 90	1,090 $\pm$ 200	1.63 $\pm$ 0.13	22 $\pm$ 2
	1:1	2,120 $\pm$ 110	2,860 $\pm$ 220	2.35 $\pm$ 0.15	25 $\pm$ 1
	RPMI	1,780 $\pm$ 110	2,700 $\pm$ 240	2.52 $\pm$ 0.20***	22 $\pm$ 1
<u>Day 4-5^b</u>	Eagle's	2,520 $\pm$ 90	530 $\pm$ 200	1.21 $\pm$ 0.09	15 $\pm$ 1
	RPMI	4,100 $\pm$ 130	3,900 $\pm$ 570	1.95 $\pm$ 0.15***	17 $\pm$ 2

Aliquots of CBA and (CBA $\times$ A) F_1 lymph node cells from initial cell suspensions were spun down and resuspended at a concentration of 2.50 million/ml in either RPMI or Eagle's medium or a medium prepared from a 1:1 mixture of these two media. In each case the medium was supplemented with 10% FCS. Cultures were incubated and labelled after 48, 72 or 96 hrs for 16 hrs.

a = Cultures were refed with 0.5 ml of appropriate medium after 48 hrs.

b = " " " " " " " 48 and 91 hrs.

+ = cp5m/100 microl of culture

++ Asterisks refer to the significance of the difference between cultures done with RPMI and cultures done with Eagle's medium.

reported in this thesis cultures were labelled with 1.0 - 1.4 microCi H-3 thymidine to give a final thymidine concentration of 6-8 nmoles/ml. However, the last batch of FCS used in these studies supported generally higher levels of DNA synthesis than did previous batches and for this serum the thymidine concentration was increased to 12 nmoles/ml for cultures also containing mouse serum, and to 30 nmoles/ml for cultures without mouse serum. In this way even the most active cultures in an experiment did not incorporate more than 20% of the total thymidine added.

5. Culture medium.

(a) Basic medium.

Two media were tested for their ability to support MLC reactions, RPMI-1640 and Eagle's MEM, both buffered with bicarbonate. The superiority of the former medium is shown in the experiment in Table 4.

The results show that cultures in RPMI + 10% FCS give considerably better MLC responses than those in Eagle's medium + 10% FCS, the difference being increasingly significant the longer the culture period. A 1:1 mixture of these two media gave somewhat lower indices than found with RPMI alone, but the differences were not significant at the 5% level. Interestingly, there was no apparent difference between cell survivals in control cultures in these media, suggesting that non-dividing cells could survive equally well in each medium.

For the work described in this thesis RPMI 1640 was used almost exclusively; in a very few experiments a 1:1 mixture of RPMI 1640 with Eagle's medium was used.

(b) Need for refeeding.

In the technique described by Festenstein (315) mouse lymphocyte cultures were refed where appropriate after 2 days and again after 4 days of culture. Since this would be rather difficult for cultures in microtest plates the importance of refeeding was studied in two

TABLE 5. A study of the necessity of refeeding mixed lymphocyte cultures.

	Mean background in unmixed cultures ⁺	Stimulation in mixed cultures ⁺	Index ⁺⁺	Survival of CBA cells ⁺⁺ (%)
<u>Experiment 1</u>				
Cultures refed	2,840 $\pm$ 195	2,270 $\pm$ 290	1.80 $\pm$ 0.14	10.4 $\pm$ 1.2
Cultures not refed	3,330 $\pm$ 105	1,530 $\pm$ 140	1.46 $\pm$ 0.05*	12.0 $\pm$ 0.4 ^{NS}
<u>Experiment 2</u>				
Cultures refed	2,330 $\pm$ 55	1,910 $\pm$ 260	1.82 $\pm$ 0.12	18.0 $\pm$ 2.3
Cultures not refed	2,190 $\pm$ 110	1,660 $\pm$ 370	1.76 $\pm$ 0.18 ^{NS}	21.0 $\pm$ 1.0 ^{NS}

CBA and (CBA $\times$ A) F_1 lymph node cells were cultured either alone or as a 1:1 mixture in tubes at a concentration of 2.5 million/ml (expt. 1) or 3.0 million/ml (expt. 2). The initial volume of cultures was 0.50 ml. After 48 and 90 hrs half the cultures in each experiment were refed with 0.50 ml of fresh medium as described in "Materials and Methods". Cultures were labelled with thymidine at 96 hrs for a further 18 hrs.

+ cp5m/100 microl of culture.

++ Significance tests were done on the difference between cultures which were refed and those which were not.

experiments in tubes. These are shown in Table 5.

In one experiment refeeding of cultures gave a significant improvement of MLC reactivity; in the second experiment a slight but insignificant improvement occurred. Thus, although refeeding was beneficial it was not essential, and because of the labour involved and the extra expense of medium no attempt was made to refeed cultures after adaptation of the technique for use in microtest plates (see section 6d of this chapter).

(c) Use of HEPES buffer.

When preparing cultures for microtest plates the volumes of cell suspensions handled are often so small in relation to their surface area that a rapid loss of carbon dioxide from a bicarbonate buffer system can occur, causing increased pH. High pH's have been found to cause reduced MLC responses (386). Furthermore, because the change in pH depends on the ratio of surface area to volume, different suspensions would be likely to end up with different pH values. To avoid such deleterious effects the use of the non-volatile buffer HEPES was investigated. The use of this buffer has been shown to improve the growth of various mammalian cells in vitro (387), and it is now widely used at concentrations ranging from 5 to 50 mM for the culture of lymphocytes. Since bicarbonate is needed for growth of lymphocytes in culture, (386,388,389), HEPES can be used as the major buffer component, cultures being incubated in CO₂ to maintain both bicarbonate levels and the secondary bicarbonate-CO₂ buffer system.

To investigate the use of HEPES for mouse MLCs the experiment described in Table 6 was performed.

At neither concentration of HEPES does the MLC index differ significantly from that obtained in the absence of HEPES. However, although no further experiments have been performed to check this, it does seem that 20 mM HEPES is somewhat toxic to mouse lymphocytes responding in MLC. Hence, HEPES was routinely used at a concentration

TABLE 6. Use of HEPES for mouse MLCs.

Concn. of HEPES (mM)	Mean background ⁺ of unmixed cultures	Stimulation in ⁺ mixed cultures	Index	CBA cell survival (%)
0	420 $\pm$ 18	2,320 $\pm$ 80	6.5 $\pm$ 0.4	19 $\pm$ 2
10	440 $\pm$ 18	2,870 $\pm$ 60	7.5 $\pm$ 0.4	22 $\pm$ 1
20	420 $\pm$ 11	2,060 $\pm$ 150	6.0 $\pm$ 0.4	24 $\pm$ 1

Appropriate volumes of a 200 mM stock solution of HEPES in RPMI at pH 7.2 were added to portions of CBA and (CBA x DBA/2) F_1 cell cultures in CM containing 10% FCS. The cells were incubated both on their own and as a 1 : 1 mixture in a microtest plate for 78 hrs, then labelled with H-3 thymidine for 16 hrs.

+ cp5m/50 microlitres of culture.

TABLE 7. The effect of FCS concentration on mouse MLC responses.

FCS concn.	Mean background ⁺ in unmixed cultures	Stimulation in ⁺ mixed cultures	Index	CBA cell survival %	% error in index
<u>Experiment 1</u>					
2%	8 ± 4	30 ± 7	-	7 ± 1	±50
5%	174 ± 26	79 ± 57	1.45 ± 0.36	19 ± 2	25.2
7½%	550 ± 49	270 ± 97	1.50 ± 0.20	22 ± 2	13.5
10%	1,160 ± 43	960 ± 57	1.82 ± 0.07	28 ± 1	4.1
<u>Experiment 2</u>					
10%	360 ± 22	770 ± 70	3.2 ± 0.3	25 ± 1	8.7
15%	460 ± 24	1,450 ± 170	4.3 ± 0.5	30 ± 2	10.3
20%	380 ± 28	1,800 ± 100	5.7 ± 0.5	26 ± 1	8.7
30%	300 ± 14	1,240 ± 35	5.1 ± 0.3	28 ± 1	5.0

In experiment 1 CBA and (CBA x A)F₁ lymph node cells were washed 3 times and resuspended in CM containing 2% FCS. To 4 equal aliquots of the two suspensions appropriate amounts of CM and FCS were added so as to make all cell concentrations 2.50 million/ml and to give the four serum concentrations shown. Mixed and unmixed cultures were dispensed into tubes and incubated 72 hrs before labelling with H-3 thymidine for 17 hrs. Cultures were refed after 47 hrs with medium containing the appropriate amount of FCS.

Experiment 2 followed a similar design, cells being resuspended initially in CM containing 10 mM HEPES and 10% FCS. CM and FCS were added so as to give final cell concentration of 5.0 million/ml and 200 microl samples of mixed and unmixed cultures dispensed into microtest plates. Cultures were labelled after 75 hrs for 17 hrs.

Different batches of FCS were used in these two experiments.

⁺ In experiment 1 figures are cp5m/100 microl.

In experiment 2 figures are cp5m/50 microl.

of 15 mM (at lower concentrations than 10 mM the buffering capacity is too low to be satisfactory).

(d) FCS concentration.

For notes on the selection of batches of FCS see "Materials and Methods". The effect of FCS concentration on mouse MLC responses is shown in Table 7.

The first experiment shows that when serum concentrations lower than 10% are used there is a sharp decrease in background transformation. This is partly due to a decrease in cell survival, but the fact that cell survival decreases much less than the mean background transformation suggests that dividing cells have a greater requirement for serum in the medium than non-dividing cells. The fall in the index of transformation suggests that MLC responding cells may have more stringent serum requirements than those cells contributing to background transformation

The conclusions from the first experiment can be extended to the second experiment, where increasing the serum concentration to 20% led to a further improvement of MLC response. At a serum concentration of 30% there was, however, decreased stimulation in mixed cultures and even at 20% serum the background transformation was reduced, showing that high concentrations of FCS can be toxic to mouse cells. For this reason, and for reason of economy, a concentration of 10% FCS was used routinely for MLC, although for cultures containing mouse serum it was found advantageous to use a concentration of 20% FCS (see Chapter 4, Section 2). It must be stressed that the experiments described in this section used only a single batch of FCS in each case. Different batches of FCS may well have somewhat different nutritional characteristics, but with all batches of FCS used a concentration of 10% was found to give satisfactory responses.

TABLE 8. Comparison of MLCs done in glass and plastic tubes.

Tube	Internal diam (mm)	Height (mm)	Mean background ⁺ of unmixed cultures	Stimulation in ⁺ mixed cultures	Index	Mean % error ⁺⁺	CBA cell survival %
Glass	9.0	48.3	1,250 $\pm$ 74	1,300 $\pm$ 180	2.04 $\pm$ 0.18	7.1	12.7 $\pm$ 1.1
Plastic	9.3	44.2	2,740 $\pm$ 165	2,240 $\pm$ 260	1.82 $\pm$ 0.13	6.7	19.0 $\pm$ 0.8

CBA and (CBA $\times$ A)₁F₁ lymph node cell suspensions in CM/10% FCS were adjusted to 2.5 million/ml and both unmixed and mixed cultures were dispensed into glass or plastic culture tubes. 10 replicate tubes were used so as to get a reliable estimate of the coefficient of variation. Cultures were refed after 50 hrs with 0.5 ml of fresh medium, and labelled after 75 hrs with H-3 thymidine for a further 16 hrs.

+ cp5m/100 microl of culture.

++ The mean % error was calculated from the formula

$$\text{Mean \% error} = 100 \times \sqrt{\frac{C_{\text{CBA}}^2 + C_{\text{CBA} \times \text{A}}^2 + C_{\text{M}}^2}{3}}$$

where the C values are the coefficients of variation in CBA, (CBA $\times$ A)₁F₁ and mixed cultures.

6. Culture vessel used.

(a) Glass v. plastic tubes.

A comparison was made between flat-bottomed sterile plastic tubes supplied by Staines Laboratories Ltd. and flat-bottomed glass tubes of almost exactly the same internal diameter and height (see Table 8) supplied by Johnson and Jorgensen Ltd, England. The glass tubes were washed thoroughly with distilled water and sterilised in a hot air oven; the plastic tubes were sterilised by the manufacturers. The results of MLCs done with the same cell suspensions in these two types of tube are shown in Table 8.

The experiment showed that although a similar index of transformation and variation between replicates was obtained with the two types of tube, cells in glass tubes had much lower background transformation than those in plastic tubes. This was due to the lower cell survival in the glass tubes. Similar results have been found by Bain and Lowenstein (390). It is very unlikely that the small differences in physical dimensions between the two tubes account for the large difference in background transformation. The slight concave curvature of the bottoms of Staines Laboratories' E2R tubes is a complicating factor here, the effective surface area at the bottoms of these tubes being less than expected from the tube diameter (see Section 6f). Consequently, one explanation of the poorer performance of cells in glass tubes is that the cell concentration was sub-optimal (Section 6). An alternative explanation is that alkaline and other materials which continue to leach out of glass even after prolonged washing adversely affected the cultured cells, although there is no noticeable difference in pH between cultures in the two containers. It should be possible to differentiate between these two possibilities by studying dose response curves in the two types of tube. However, during the course of this experiment the difficulties of handling the glass vials, including the need to sterilise them, became clear and, as they had no apparent advantages for

TABLE 9. Testing different batches of plastic tubes.

Tube		Mean background ⁺ in unmixed cultures	Stimulation in ⁺ mixed cultures	Index	CBA cell survival %
2 ml (E2R) Diam. 9.3 mm Height 44.2 mm	Batch 1	5,640 $\pm$ 290	6,070 $\pm$ 680	2.07 $\pm$ 0.15	28 $\pm$ 3
	Batch 2	4,000 $\pm$ 280	5,180 $\pm$ 330	2.30 $\pm$ 0.17	23 $\pm$ 4
	Batch 3	4,040 $\pm$ 190	4,890 $\pm$ 360	2.21 $\pm$ 0.13	20 $\pm$ 2
2½ ml (E2.5R) Diam. 9.4 mm Height 52.2 mm	Batch 1	2,780 $\pm$ 190	4,400 $\pm$ 520	2.58 $\pm$ 0.24	16 $\pm$ 2
	Batch 2	2,040 $\pm$ 200	3,640 $\pm$ 610	2.78 $\pm$ 0.39	14 $\pm$ 2

CBA and (CBA x A)₁F₁ lymph node cell suspensions were adjusted to 2.50 million viable cells/ml in CM/10% FCS. Control and mixed cultures were dispensed into the different batches of tubes. Cultures were refed after 52 hrs and labelled after 72 hrs with H-3 thymidine for 16 hrs.

+ cp³m/100 microl of culture

cell culture, plastic tubes were the only tubes for subsequent work.

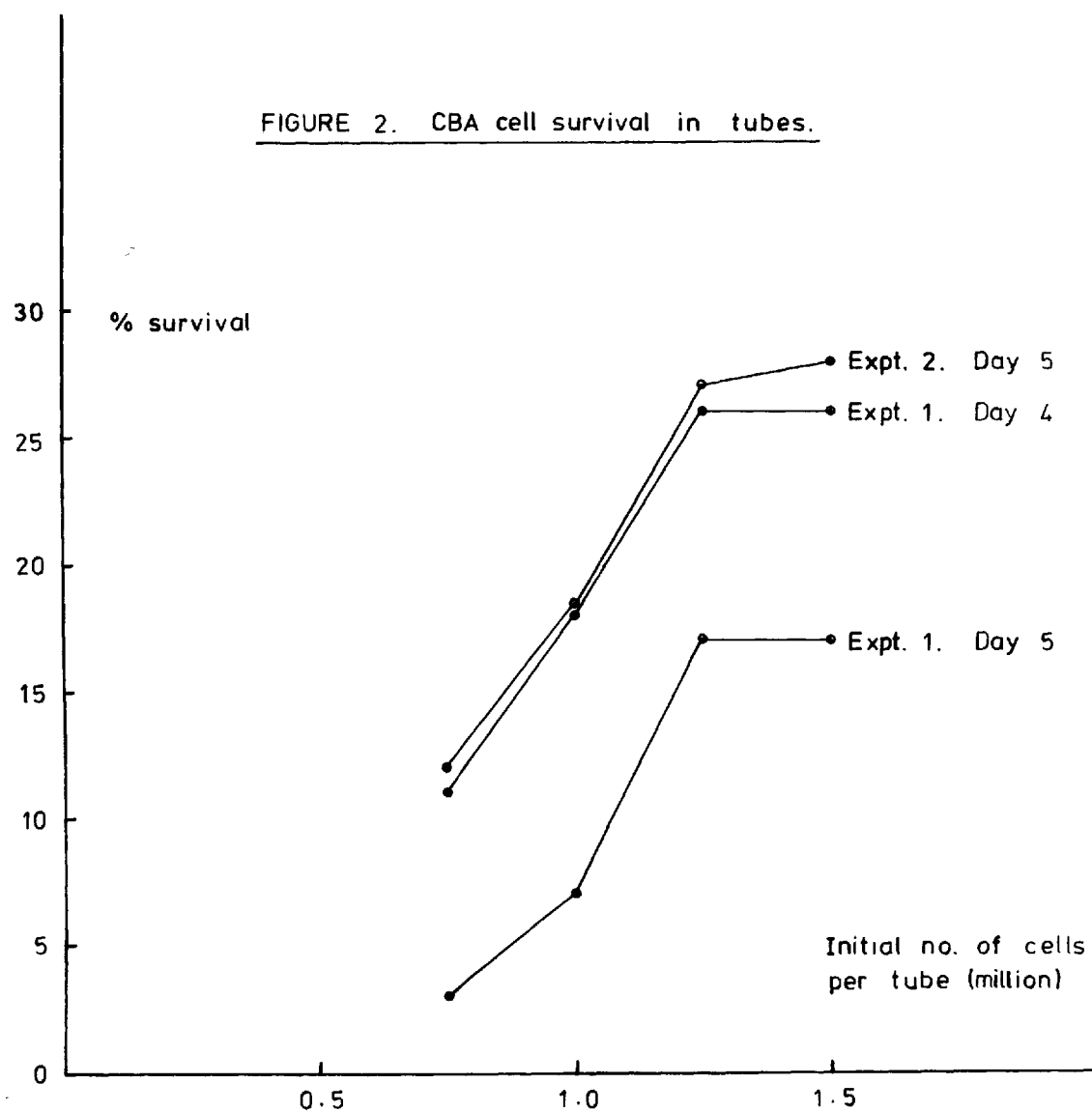
(b) Different batches of plastic tubes.

3 batches of 2 ml tubes (Staines Laboratories' E2R) and 2 batches of $2\frac{1}{2}$ ml tubes (Staines Laboratories' E2.5R), both flat-bottomed, were obtained. These tubes have closely similar diameters. Their ability to support mouse MLC responses was tested and the results are shown in Table 9.

Cultures done in $2\frac{1}{2}$ ml tubes had significantly lower backgrounds ($P = 0.04$) and cell survivals ($P = 0.04$) than in the 2 ml tubes. The easiest interpretation of these results is that the $2\frac{1}{2}$ ml tubes contained a toxic material which may also have been present in 2 ml tubes but, if so, at lower concentration. A number of workers have found that plastic tubes can be toxic to cell cultures, the toxicity apparently being due to residues of ethylene oxide gas which manufacturers use for sterilisation. In a detailed study Bain and Lowenstein (390) found that when tubes had been stored for a few months they lost their initial toxicity. Tubes from the same manufacturer which had not been subjected to chemical sterilisation or which had been sterilised by ultraviolet light were found to be non-toxic. A decrease of toxicity of some batches of chemically sterilised tubes has also been found by du Bois et al (391) and by Knight (392), the latter worker using tubes from Staines Laboratories. It thus seems likely that the variation in DNA synthesis of cells in the different batches of tubes tested in the experiment of Table 9 is at least partly due to varying residues of ethylene oxide gas which Staines Laboratories used for sterilising their tubes.

A second factor which may have been important is the slightly different geometry of the two types of tubes. The $2\frac{1}{2}$ ml tubes are approx. 20% taller than the 2 ml tubes and, more important, have almost perfectly flat bottoms, whereas the 2 ml tubes have slightly concave bottoms. Consequently, the bottoms of the two tubes have

FIGURE 2. CBA cell survival in tubes.



slightly different effective surface areas (see Section 6f) and the lower background and cell survival in $2\frac{1}{2}$ ml tubes could be due to their having a sub-optimal cell concentration.

Attempts to investigate these two factors were not made, and 2 ml Staines E2R tubes, kept in storage for at least two months prior to use, were employed for all culture work done in tubes.

(c) Optimal cell concentration in plastic tubes.

The effect of initial cell concentration on the degree of MLC reaction in tubes is shown in Table 10. In Figure 2 the effect on cell survival is shown.

Figure 2 shows that CBA lymph node cell survival becomes optimal when the initial cell concentration reaches 1.25 million viable cells/tube; with lower starting concentrations the percentage of cells surviving 4 or 5 days later decreases steeply; with the higher starting concentration of 1.5 million cells/tube conditions for cell survival are very similar to those at 1.25 million/ml. Clearly, mouse lymphocytes require for survival the presence of other cells around them - such effects are usually referred to as "feeder effects".

A similar situation is found when the performance of cells in cultures is measured by DNA synthesis (Table 10). The mean background DNA synthesis drops drastically when the initial cell concentration is below 1.25 million/ml. The sequential decrease in index of transformation in experiment 1 suggests that those cells which respond in MLC are particularly dependent on feeder effects. At lower cell concentrations the variation between replicate tubes can become very large as shown by the increase in percentage error in the index to about 20% in tubes with low cell concentration. Only a small fraction of this increased error is due to an increase in the error of scintillation counting of samples with lower levels of radioactivity. The increased error is presumably caused by the fact that at lower cell concentrations one is no longer working in the plateau region of the feeder

TABLE 10. The effect of initial cell concentration on MLC response in tubes.

Initial no. of cells/tube (million)	Mean background* of unmixed cultures	Stimulation in* mixed cultures	Index	% error in index	CBA cell survival (%)
<u>Experiment 1</u>					
<u>Day 3-4</u>					
0.75	156 $\pm$ 11	58 $\pm$ 45	1.37 $\pm$ 0.30	21.7	11 $\pm$ 4
1.00	770 $\pm$ 56	420 $\pm$ 81	1.54 $\pm$ 0.14	8.8	18 $\pm$ 2
1.25	1,280 $\pm$ 48	950 $\pm$ 188	1.74 $\pm$ 0.16	9.0	26 $\pm$ 1
1.50	1,450 $\pm$ 50	1,200 $\pm$ 165	1.76 $\pm$ 0.12	7.1	26 $\pm$ 1
<u>Day 4-5</u>					
0.75	446 $\pm$ 52	54 $\pm$ 108	1.12 $\pm$ 0.25	22.3	3 $\pm$ 0.2
1.00	1,540 $\pm$ 96	690 $\pm$ 56	1.45 $\pm$ 0.10	6.7	8 $\pm$ 0.5
1.25	2,200 $\pm$ 176	1,890 $\pm$ 252	1.86 $\pm$ 0.17	9.1	17 $\pm$ 2
1.50	2,200 $\pm$ 141	1,540 $\pm$ 307	1.70 $\pm$ 0.17	10.1	17 $\pm$ 1
<u>Experiment 2</u>					
<u>Day 4-5</u>					
0.75	530 $\pm$ 76	950 $\pm$ 185	2.79 $\pm$ 0.51	18.3	12 $\pm$ 2
1.00	690 $\pm$ 59	590 $\pm$ 257	1.86 $\pm$ 0.40	21.3	18 $\pm$ 2
1.25	2,300 $\pm$ 125	2,620 $\pm$ 264	2.14 $\pm$ 0.15	7.2	27 $\pm$ 3
1.50	3,740 $\pm$ 178	2,750 $\pm$ 384	1.74 $\pm$ 0.12	7.1	28 $\pm$ 2

In experiment 1 CBA and (CBA $\times$ A)F₁ lymph node cell suspensions were prepared. These two suspensions were diluted with CM/10% FCS to give various cell concentrations and 0.50 ml aliquots of unmixed and mixed cultures were dispensed into tubes. Cultures were refed after 48 and 91 hrs with 0.50 ml fresh medium and labelled after 72 or 96 hrs with H-3 thymidine for 16 hrs. Experiment 2 was performed in an exactly analogous way except that cultures were labelled at 96 hrs only.

* cp5m/100 microl of culture

TABLE 11. Is washing of cells after labelling necessary ?

	Mean background+ of unmixed cultures	Stimulation in+ mixed cultures	Index of transformation	CBA cell survival (%)	Supernatant+ counts
A	1,160 $\pm$ 60	1,120 $\pm$ 150	1.96 $\pm$ 0.16	39 $\pm$ 1%	160
B	1,110 $\pm$ 40	1,140 $\pm$ 200	2.05 $\pm$ 0.19	31 $\pm$ 2%	130

0.5 ml of unmixed and mixed CBA and (CBA x A)_F₁ lymph node cell suspensions at 3.0 million/ml in CM/10% FCS were dispensed into tubes. After 73 hrs they were labelled with H³ TdR for 16 hrs. Cultures in group A were cooled in ice for 1 hr and then harvested by mixing and removing 100 microl samples directly onto discs. While group A cultures were in ice, group B cultures were washed once with 2 ml of cold PBS, the volume reduced to 0.50 ml and 100 microl samples removed onto discs. After taking samples for scintillation counting all cultures were fixed with glutaraldehyde and cell counts done on coded tubes. Discs were allowed to dry for about 2 hrs at room temperature, then extracted as described in Materials and Methods.

+ cp5m/100 microl of culture.

effect. In the plateau region the feeder effect is relatively constant so that any tendency for a variation in cell survival in different tubes will not be magnified by resulting differences in feeder effects. In the second experiment the increased errors at low initial cell concentration probably obscured a decline in the index of transformation, but the large decrease in background DNA synthesis at lower cell concentration is again clearly demonstrated.

(d) Use of microtest plates for culture.

Having shown that refeeding of cultured cells was not essential (Section 5b) the second problem to be solved before the adaptation of MLC technique to microtest plates could be attempted was whether it was necessary to wash cells after labelling with thymidine before placing samples on discs for TCA extraction. The experiment shown in Table 11 was performed.

There is no significant difference between the two estimates of DNA synthesis and MLC responsiveness. Furthermore, the supernatant counts are only slightly higher for unwashed cultures, showing that the TCA/PCA washing of the discs removes all free thymidine effectively. Interestingly, there is a decreased cell survival in tubes which had been washed with PBS. Since there is no significant decrease in mean background DNA synthesis the loss of viability must have occurred during the PBS washing procedure. Thus there is a second advantage in harvesting cells directly.

Having shown that the post-labelling washing of cells was unnecessary the suitability of Falcon microtest II flat-bottomed plates for mouse MLC cultures was tested. These plates were chosen since they are specially made for cell culture and have been used by a number of workers for this purpose, in particular by Hellström et al (393) for their experiments on neonatally-induced tolerance.

TABLE 12. Comparison of Falcon microtest plates and Staines E2R tubes at optimal cell concentrations.

	Background DNA synthesis per million initial cells ⁺		CBA cell survival %	Index of transformation ⁺
	CBA	(CBA x A)F ₁		
Plates	3,720 $\pm$ 60	3,120 $\pm$ 130	25.6 $\pm$ 2.4	2.36 $\pm$ 0.05
Tubes	3,800 $\pm$ 50	3,360 $\pm$ 230	27.6 $\pm$ 2.0	1.81 $\pm$ 0.07

+ Figures are based on the results of Experiment 1, Table 13, Section 6e.

Table 12 shows that at the optimal cell concentration in microtest plates (1.0 million cells/well; Section 6e) the background DNA synthesis by mouse lymph node cells and their % cell survival were almost identical to those found in tubes also containing an optimal concentration of cells (1.50 million/tube; Section 6c). It seems that the conditions for cell survival are closely similar in the two types of container. For MLC response, however, microplates seem to have a clear advantage, the index of transformation being significantly higher ($P < 0.001$) than in tubes.

Clearly microplates are quite suitable for mouse MLC cultures. They have the additional advantages over tubes of

- (i) much greater ease of handling
- (ii) requiring less incubator space
- (iii) greater economy of both cells and medium.

However, as work with microplates proceeded two problems were encountered. Firstly, the experiment shown in Table 11 demonstrated that it was not necessary to prewash labelled cells before harvesting. It was later found that this conclusion was correct only when using a batch of FCS supporting high levels of DNA synthesis. In subsequent work a batch of serum was used which only allowed about

one-tenth the amount of DNA synthesis of the serum used previously. This serum was in no way toxic as judged by cell survival but it seemed to be deficient in factors promoting DNA synthesis. As a result, the supernatant counts retained on discs after TCA washing were of a similar order to those incorporated into cellular DNA. Consequently estimation of nett DNA synthesis by subtraction of supernatant counts from total culture counts led to very high percentage errors. To overcome this problem it was necessary to remove culture supernatant from a labelled plate by holding the plate upside down over a sink and giving it a sharp jerk. This procedure removes about 90% of supernatant medium from the wells, the remaining supernatant and all the cells being retained at the bottom of the wells by surface tension. After adding 200 microl of fresh medium the cultures could be harvested in the usual way. This method reduces the supernatant counts to satisfactorily low levels when using batches of FCS with low ability to support DNA synthesis, but wherever possible was avoided by using batches of FCS allowing high DNA synthesis because the dumping process has the disadvantage of being time-consuming, introducing extra errors into the harvesting process and, unless done very carefully, can lead to radioactive contamination of surrounding benches.

Secondly, it became apparent that systematic variation is found in different parts of the plate. The two effects noticed were:

- (i) one half of the plate would occasionally have lower levels of DNA synthesis than the other;
- (ii) wells on the outside of the plate sometimes allowed higher levels of DNA synthesis than those on the inside. A similar finding has been reported by Sanderson (394).

To overcome the danger of a systematic variation between cultures a random code was devised: the 4 replicates of a given cell suspension were placed one in each quadrant of the plate so that

FIGURE 3. Cell survival in microtest plates

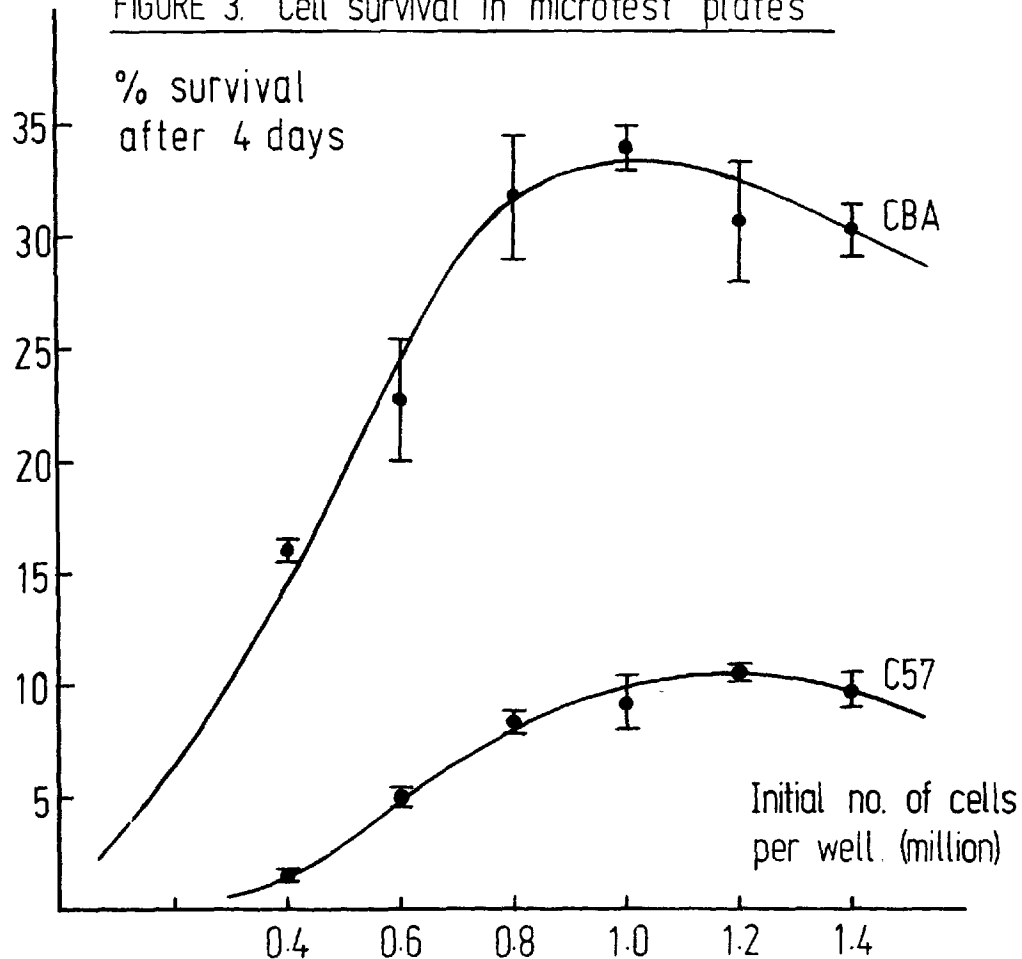
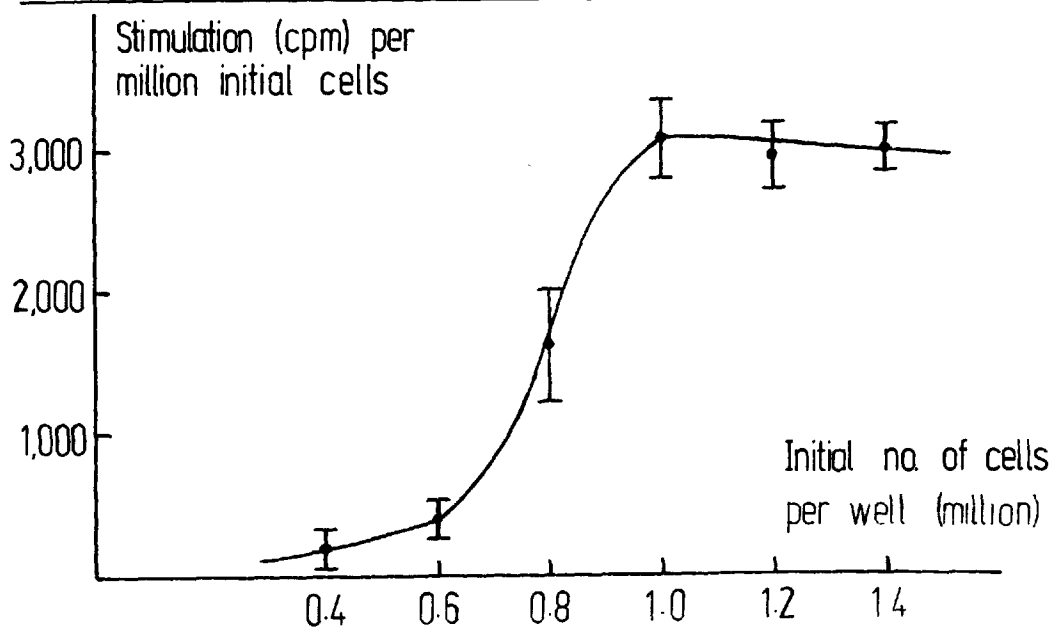


FIGURE 4. Response in CBA v. CBAx_A cultures in microtest plates.



each side of the plate had 2 representative samples of each cell suspension. Within each quadrant cultures were plated according to a pre-determined random distribution. This largely overcomes errors similar to those of type (ii) above. In those occasional plates where a consistent difference in levels of DNA synthesis was found between the two sides of the plate the mean ratio of counts in each quadrant was calculated and individual cultures corrected for this. The standard error between replicate cultures, then containing only a random variation, would then be calculated in the usual way.

(e) Optimal cell concentration in microtest plates.

The results of two experiments in microtest plates (Table 13) gave results very similar to those obtained using tubes (Section 6c). Figure 3 shows the increase in percentage cell survival that occurs for two strains of cell as the initial cell concentration is increased. For both cells an optimal initial cell concentration is found (at 0.8-1.0 million cells/well for CBA and at about 1.2 million cells/well for C57B1) at which feeder effects have become maximal; at higher concentrations the percentage cell survival shows evidence of a decline.

Figure 4 shows the stimulation per million initial cells in mixed cultures of CBA and (CBA x A)_{F₁} cells as a function of initial cell concentration. With less than about 0.6 million cells per well only a negligible stimulation is found in mixed cultures. There is then a phase of rapidly increasing MLC responsiveness until a plateau is reached with about 1.0 million cells/well. Comparing Figures 3 and 4 it appears that mouse lymph node cells will not respond in MLC unless the feeder effects in culture are close to optimal, since although at a concentration of 0.6 million cells/well there is a moderate feeder effect there is almost no MLC response. It was not possible to overcome the need for high cell concentrations by using a

TABLE 13. Effect of initial cell concentration on MLC responses in microtest plates.

No. of cells/well (million)	Background DNA synthesis in:			CBA v. (CBA x A)F ₁ mixed cultures		
	CBA	(CBA x A)F ₁	C57B1	Stimulation	Index	% error in index
Experiment 1						
0.40	66 ± 14	22 ± 12	ND	6 ± 10	1.14 ± 0.25	21.9
0.60	268 ± 50	140 ± 28		380 ± 48	2.83 ± 0.44	15.4
0.75	1,060 ± 86	580 ± 60		1,860 ± 100	3.24 ± 0.24	7.3
1.00	3,720 ± 60	3,120 ± 130		4,660 ± 100	2.36 ± 0.05	2.4
Tube (1.50 million)	5,700 ± 76	5,040 ± 350		4,340 ± 150	1.81 ± 0.07	4.2
Experiment 2						
(a) 10% FCS						
0.40	370 ± 25	280 ± 30	50 ± 30	170 ± 140	1.53 ± 0.42	27.0
0.60	2,170 ± 210	1,250 ± 110	280 ± 70	580 ± 210	1.34 ± 0.14	10.0
0.80	6,860 ± 490	2,760 ± 120	1,430 ± 120	3,250 ± 800	1.67 ± 0.18	11.0
1.00	10,200 ± 380	4,780 ± 330	3,410 ± 460	7,700 ± 710	2.03 ± 0.11	5.5
1.20	13,600 ± 660	7,680 ± 250	4,570 ± 110	8,900 ± 700	1.83 ± 0.08	4.5
1.40	14,400 ± 830	8,790 ± 440	4,800 ± 135	10,600 ± 560	1.93 ± 0.08	4.2
(b) 20% FCS						
0.60	2,040 ± 160	1,010 ± 25	ND	600 ± 150	1.40 ± 0.11	8.0
0.80	6,410 ± 260	2,450 ± 270		5,100 ± 770	2.15 ± 0.19	8.9
1.00	12,900 ± 1,060	5,000 ± 400		7,450 ± 770	1.83 ± 0.13	7.2
1.20	14,500 ± 250	7,650 ± 550		10,900 ± 950	1.99 ± 0.10	4.9

In experiment 1 CBA and (CBA x A)F₁ lymph node cells were adjusted to 5.0 million/ml in CM/10% FCS. Unmixed and mixed cell suspensions were diluted with CM/10% FCS to various concentrations and 200 microl aliquots dispensed into the wells of a microtest plate. 500 microl aliquots of cell suspensions at 3.0 M/ml were also dispensed into plastic tubes. Cultures were labelled with H-3 thymidine after 98 hrs for 16 hrs, twice as much thymidine being given to cultures in tubes as to cultures in microtest plates.

In experiment 2 CBA, (CBA x A)F₁ and C57B1 lymph node cells were cultured in CMH containing either 10 or 20% FCS. Unmixed and mixed cultures were diluted to various concentrations in CMH containing the appropriate amount of FCS and 200 microl dispensed into the wells of microtest plates. After 73 hrs cultures were labelled for 16 hrs with H-3 thymidine.

All counts are expressed as cp5m/100 microl of culture for microtest plates and cp5m/250 microl of culture for tubes.

higher concentration of FCS. As shown in experiment 2 (Table 13) the results with 20% FCS followed exactly the same pattern as with 10% FCS.

Finally, as observed with tube cultures, there is a decrease in % error in microtest-plate cultures as the cell concentration increases.

(f) Significance of the optimal cell concentrations.

The cell concentration at which feeder effects and MLC responses become maximal is about 1.25 million cells per tube and about 1.0 million cells per microtest well. What is the significance of these concentrations ?

Measurements of cells in a haemocytometer showed that the great majority of cells found in the lymph node cell suspensions used for setting up MLCs were of a fairly uniform size of 6.5 micron (small lymphocytes). Only occasional medium size cells, c. 9.5 micron in diameter, were found while large lymphocytes, diameter c. 12.5 micron, were even less common.

Assuming that lymphocytes in culture pack together in the most efficient way the total area occupied by N cells of diameter d would be $Nd^2 \sin 60^\circ$. The mean diameter of wells in Falcon microtest II plates is 6.4 mm. Thus the number of cells required to form a complete monolayer on the bottom of such wells is
$$\frac{\pi \times (6.4^2 \times 10^{-3})^2}{4 \times d^2 \sin 60^\circ}$$

Neglecting the occasional large cells in cell suspensions this gives

$$N = \frac{\pi \times (6.4 \times 10^{-3})^2}{4 \times (6.5 \times 10^{-6})^2 \times \sin 60^\circ} = 0.88 \times 10^6$$

The same calculation for plastic tubes (diam. 9.3 mm) gives

$$N = \frac{\pi \times (9.3 \times 10^{-3})^2}{4 \times (6.5 \times 10^{-6})^2 \times \sin 60^\circ} = 1.86 \times 10^6$$

The number of cells calculated to be required for monolayer formation in microtest wells is very close to that found in the experiments above to be required for optimal cell survival and MLC response.

That almost perfect cell monolayers are formed at such concentrations was confirmed by examining microplate cultures with an inverting microscope. At the point at which monolayer formation becomes complete, cell-cell contact is maximised and it is at this point that the ability of cells to survive in culture and respond to alloantigens becomes maximal. Adding further cells to the cultures leads, if anything, to a decrease in cell survival (Figure 3), suggesting that even a slight layering of cells on top of each other causes an increased death rate due to "suffocation", although the alternative explanation that the larger numbers of cells causes depletion of nutrients from the medium cannot be ruled out here.

In tubes the situation is slightly different. The calculated number of cells needed for monolayer formation (1.86×10^6) is significantly greater than the number of cells required for a maximal feeder effect (1.25×10^6). The reason for this is almost certainly due to the fact that the bottoms of the Staines Laboratories tubes are not completely flat. They have a slight concave curvature causing cells to gravitate outwards to some extent from the centre of the tube; the effective surface area of these tubes is thus reduced. A similar effect has been found by Thorpe (395), who measured mitogen responses of varying numbers of mouse lymphocytes in flat-bottomed and round-bottomed microtest plates. In the flat-bottomed plates (of a different make to the plates used here) mouse lymphocytes showed optimal PHA responses when there were about 1.2 million cells per well, whereas in round-bottomed plates optimal responses occurred at about 0.6 million cells per well. Gravitation of cells towards the centre of round-bottomed wells allows an initial small number of cells to come into intimate contact with each other.

7. The use of irradiated stimulator cells.

(a) The effect of irradiated cells on feeder effects.

It was shown in Section 6 that feeder effects become optimal in

TABLE 14. Testing the ability of irradiated cells to supply feeder effects.A. Live CBA v. live (CBA x A)_{F1} cultures.

Total no. of cells per well (million)	Background in unmixed cultures		Mixed cultures	Stimulation in mixed cultures	Index
	CBA	(CBA x A) _{F1}			
2.0	22,200 ± 1,000	ND	13,900 ± 2,400	8,700 ± 2,400	2.7 ± 0.5
1.0	4,480 ± 470	5,890 ± 240			
0.5	180 ± 50	ND			

B. Live CBA v. irradiated cultures.

Number of cells per well (million)			CBA v. CBA _x	[CBA v. (CBA x A) _{F1}] _x	Stimulation	Index
Total	Live	Irradiated				
1.5	1.0	0.5	3,810 ± 300	11,400 ± 1,000	7,600 ± 1,000	3.0 ± 0.4
1.0	0.5	0.5	80 ± 80	320 ± 80	240 ± 110	-

C. Irradiated cells alone.

No. of cells/well (million)	CBA _x	(CBA x A) _{F1} x
2.0	-20 ± 40	ND
1.0	ND	90 ± 40

CBA and (CBA x A)_{F1} lymph node cell suspensions were adjusted to either 10.0, 5.0 or 2.5 million cells/ml in CMH/15% FCS. Portions of these cell suspensions were irradiated with 1000R, spun down and resuspended in fresh medium at the same concentration. Appropriate mixed and unmixed cultures were dispensed into the wells of a microtest plate and incubated for 72 hrs before labelling with H-3 thymidine for 16 hrs. All counts are cp10m/100 microl. of culture.

microtest plates when there are 1.0 million cells/well. Whether irradiated cells are able to supply a feeder effect was studied in the experiment shown in Table 14.

Part A of the experiment, using live stimulator cells, shows the typical responses obtained with an initial cell concentration of 1.0 million cells/well and the almost negligible background cell transformation at 0.5 million cells/well due to the breakdown of feeder effects (Section 6). Part B shows how live cells behave in the presence of irradiated cells. The fact that irradiated cells are completely unable to supply any feeder effect is shown by the following observations:

- (i) The background transformation of 1.0 million CBA cells comprising 0.5 million live cells and 0.5 million irradiated cells does not differ significantly from that of 0.5 million live cells alone but differs completely from half the background transformation of 1.0 million live cells alone ($P < 0.001$); similarly the background transformation of 1.5 million CBA cells comprising 1.0 million live cells and 0.5 million irradiated cells does not differ significantly from that of 1.0 million live cells alone.
- (ii) Mixed cultures between 0.5 million live CBA cells and 0.5 million irradiated (CBA x A) F_1 give a negligible response compared to mixed cultures of 0.5 million live CBA cells and 0.5 million live (CBA x A) F_1 cells.

As it turns out, mixed cultures at a 2:1 ratio, where 1.0 million live CBA cells were mixed with 0.5 million irradiated F_1 cells, give similar responses to the normal 1:1 mixture of 0.5 million live CBA and 0.5 million live F_1 cells. Consequently, most experiments with irradiated cells were performed at a 2:1 ratio of responder:stimulator cells, using the cell concentrations described here.

A further important point emerges from this experiment. Many workers using irradiated or mitomycin-treated stimulator cells for MLC have used as their control to confirm the effectiveness of the treatment R_x v. S_x or R_m v. S_m cultures (see for example refs. 396, 397). They have concluded that zero response in such cultures indicates that the DNA synthetic capacity of the cells has been completely blocked. But the experiment above shows that when as few as half the cells in a culture have received 1000R of irradiation a negligible MLC response is obtained. This is because the killing of half of the cells by irradiation abolishes their ability to provide a feeder effect for the remaining cells, which then subsequently die. However, if such "half-killed" cells are mixed with more live cells (e.g. in an R v. S_x mixture) the apparently unresponsive cells can now respond very well. This is shown in section B of the above experiment: 0.5 million live CBA cells mixed with 0.5 million irradiated CBA cells show zero background transformation (80 ± 80), but when these are added to a further 0.5 million live CBA cells there is now a considerable background transformation ($3,810 \pm 300$).

(b) Dosage of radiation needed for complete inactivation of MLC responsive cells. (Table 15).

To test the effectiveness of irradiation, irradiated parental cells were not cultured on their own but with live F_1 cells.

In this experiment the cell survival was for some reason very low, especially for the F_1 cells (which always show lower levels of survival than CBA cells), so that background responses were very low. It is nevertheless quite clear that irradiation has reduced the MLC responsiveness of CBA cells to negligible levels compared to that of live cells. Yet with irradiation doses of less than 2000R there is evidence of residual responsiveness in the irradiated CBA cells towards (CBA x DBA/2) MLC antigens; a t-test on the

TABLE 15. Inactivation of MLC responsive cells by X-rays.A. Positive control.

Mean background in unmixed cultures	Stimulation in mixed (1:2) cultures	Stimulation per million initial CBA cells
310 $\pm$ 40	3,680 $\pm$ 130	11,140 $\pm$ 390

B. With varying doses of irradiation.

Radiation dose	(CBA x DBA/2) _x v. (CBA x DBA/2)	CBA _x v. (CBA x DBA/2)	Stimulation	Stimulation per million initial CBA cells
1000R	222 $\pm$ 37	342 $\pm$ 47	+ 120 $\pm$ 60	+ 240 $\pm$ 120
1500R	167 $\pm$ 13	277 $\pm$ 23	+ 110 $\pm$ 26	+ 220 $\pm$ 52
2000R	187 $\pm$ 30	128 $\pm$ 12	- 59 $\pm$ 32	- 118 $\pm$ 64
	CBA v. CBA _x	CBA v. (CBA x DBA/2) _x	Stimulation	
1000R	434 $\pm$ 47	10,630 $\pm$ 570	10,200 $\pm$ 570	
2000R	309 $\pm$ 27	4,530 $\pm$ 380	4,220 $\pm$ 380	

In part A ~~un~~irradiated CBA and (CBA x DBA/2)_{F₁} lymph node cells were cultured separately or mixed in a 1:2 responder:stimulator ratio. In part B the CBA and (CBA x DBA/2)_{F₁} cells were given varying amounts of irradiation and mixed in a 1:2 ratio with either live (CBA x DBA/2)_{F₁} cells (to measure whether the radiation dose had prevented P v. F₁ responses) or with live CBA cells (to measure how well the irradiated cells were able to stimulate.) Cultures were labelled after 70 hrs with H-3 thymidine for 16 hrs. All figures are cp10m/100 microl. of culture.

combined results at 1000 and 1500R shows a significant stimulation at the $P = 0.004$ level. However, in view of the low survival of the F_1 cells further experiments would be necessary to confirm the validity of this finding.

With increasing amounts of radiation the ability of cells to stimulate in MLC also declines. After 2000R F_1 cells had only half the stimulatory power of cells given only 1000R. Such effects, although usually at higher radiation doses, have been found previously by other workers (398-400), the most likely explanation being that the increasing damage to the genetic material of the cells reduces the amount of protein and hence antigen synthesis, and may also lead to a more rapid deterioration in cell membrane structure and even cell disintegration. Despite the lower stimulatory power of cells given 2000R this radiation dose is essential to ensure complete unidirectional responses. Further experiments confirming that this dose of irradiation was sufficient are summarised in Table 16. These results also confirm that F_1 cells do not "respond" to irradiated parental cells under the culture conditions used here (see introduction to this chapter).

8. The effect of temperature on MLC responses.

In the pioneering work of Festenstein on the development of techniques for mouse lymphocyte culture a temperature of $33-34^{\circ}$ was used to reduce haemolysis of red cells. All other workers have used a temperature of 37° . To test the abilities of mouse cells cultured at these two temperatures to respond in MLC the two experiments shown in Table 17 were performed. The two experiments were done with different strains and types (irradiated, not irradiated) of stimulator cells and with two different batches of FCS. Both experiments show the following points:

TABLE 16. Is 2000R sufficient to completely block MLC responsiveness ?

	$(C \times D)_x$ v. C x D	CBA_x v. C x D	Stimulation
Experiment 1	$3,700 \pm 110$	$3,160 \pm 170$	$- 540 \pm 200$
Experiment 2	$6,550 \pm 350$	$6,320 \pm 260$	$- 230 \pm 440$
Experiment 3	$2,060 \pm 190$	$2,960 \pm 310$	$+ 900 \pm 370$
	$(C \times B)_x$ v. C x B	CBA_x v. C x D	Stimulation
Experiment 4	$2,480 \pm 260$	$1,860 \pm 100$	$- 580 \pm 240$
Mean stimulation			$- 110 \pm 160$

All figures are cp10m/50 microl. of culture. $C \times D = (CBA \times DBA/2)F_1$, $C \times B = (CBA \times Balb/C)F_1$. In each case unirradiated CBA cells showed strong responses to the irradiated F_1 cells.

TABLE 17. The effect of temperature on MLC responses.Experiment 1 - Live stimulator cells.

	Temperature (°C)	Unmixed cultures		1:1 Mixed cultures	Stimulation in mixed culture	Index
		CBA	(CBA x C57B1)F ₁			
DNA synthesis ⁺	33½	12,200 ± 730	1,200 ± 80	27,700 ± 860	21,000 ± 900	4.13 ± 0.26
	37	7,140 ± 1,170	430 ± 70	20,500 ± 1,200	16,700 ± 1,300	5.42 ± 0.89
% Error between replicates	33½	6	7	3		
	37	16	16	6		
% Cell survival	33½	27.8 ± 2.4	5.6 ± 0.6	ND		
	37	12.0 ± 1.2	1.0 ± 0.2	ND		

Experiment 2 - Irradiated stimulator cells.

	Temperature	CBA v. CBA _x	CBA v. (DBA/2) _x	Stimulation by allogeneic cells	Index
DNA synthesis ⁺	33½	4,910 ± 270	32,500 ± 440	27,600 ± 520	6.61 ± 0.37
	37	800 ± 65	28,200 ± 1,360	27,400 ± 1,360	35.3 ± 3.3
% Error between replicates	33½	5.5	1.3		
	37	8.1	4.8		
% Cell survival	33½	33.6 ± 2.6	44.6 ± 3.0		
	37	6.2 ± 1.6	39.0 ± 2.8		

In experiment 1 aliquots of 1.0 million cells from mixed and unmixed cultures of CBA and (CBA x C57B1)F₁ lymph node cells in CMH/20% FCS were dispensed into two microtest plates. The plates were placed in identical desiccators gassed in an identical way and incubated in two incubators, one at 37°C, the other at 33½°C. After 96 hrs cultures were labelled with H-3 thymidine and incubated as before for a further 16 hrs. In the second experiment aliquots of 1.0 million CBA lymph node cells mixed with 0.5 million irradiated CBA or DBA/2 spleen cells in CMH/20% FCS were dispensed into microtest plates and incubated as described above. Cultures were labelled after 72 hrs for 18 hrs. In both experiments, after harvesting samples for measurement of DNA synthesis, the remaining parts of cultures were fixed with glutaraldehyde and cell counts done.

⁺ Figures for DNA synthesis are cp5m/100 microl. of culture.

- (i) The survival of unstimulated cells is very much lower at 37° . In the first experiment there was a 64% reduction in CBA cell survival at 37° compared to $33\frac{1}{2}^{\circ}$ and an 82% reduction in F_1 cell survival. In the second experiment, the survival in control cultures was 82% less at 37° than at $33\frac{1}{2}^{\circ}$. The differences in control cell survival are reflected by the differences in background transformation, the corresponding reductions at the higher temperature being 41%, 64% and 84% respectively.
- (ii) By contrast, the cell proliferation which occurs in mixed cultures occurs equally well at both temperatures. This is shown by the similar amounts of DNA synthesis which occur in mixed cultures at the two temperatures. Because of the low DNA synthesis in control cultures at 37° the index of transformation at this temperature is higher than at $33\frac{1}{2}^{\circ}$, sometimes very much so (experiment 2). The apparently elevated response at 37° is mainly an artefact of the culture conditions for, as shown in experiment 2, the cell survival in mixed cultures at 37° is very different to that in control cultures at the same temperature. As a result the conditions in these two types of culture, most importantly the degree of cell-cell contact (feeder effect - see Section 6), are vastly different, so much so that the unmixed cultures cease to be useful controls for general culture conditions in the mixed culture.
- (iii) The percentage errors between replicate wells cultured at 37° are consistently about twice the size of those between replicate wells at $33\frac{1}{2}^{\circ}$. As a result, statistical tests

performed on data obtained at the lower temperature have an approximately 2-fold higher significance level than corresponding tests on cultures incubated at 37° . This is vividly shown in experiment 2 in which the index of transformation at 37° was 35.3 ± 3.3 . This index is in fact less significant than the index of 6.61 ± 0.37 . In this particular case the exercise is academic since both responses are significant at an extremely high level. But in other cases, where for instance the reactivity of two cells was being compared, the chances of detecting a significant difference between the two reactivities would be greater if the cultures were done at $33\frac{1}{2}^{\circ}$. Many workers have made the mistake in devising MLC systems of aiming at the highest possible indices; a much more important parameter than the magnitude of the index is the percentage error involved in the measurement of this quantity.

Because of the multiple advantages of culturing mouse cells at a lower than normal temperature, all MLC work reported here was done at a temperature of $33-34^{\circ}$.

9. Type of cell used for MLC.

(a) Comparison of lymph node and spleen cells.

Table 18 shows that as far as stimulating ability is concerned spleen cells and lymph node cells are equally effective stimulators of allogeneic lymph node cells as measured by the excess counts in allogeneic compared to control mixed cultures. However, when the index of transformation is used as a measure of stimulating ability spleen cells appear to be inferior to lymph node cells. The reason is that in syngeneic cultures of lymph node v. spleen cells the background transformation is higher than in lymph node v. lymph node

TABLE 18. Comparison of the ability of lymph node and spleen cells to respond and stimulate in MLC.

	Responder cells	Stimulator cells	CBA v. CBA _x	CBA v. (CBA x C57Bl) _{F_{1x}}	Stimulation in allogeneic cultures	Index
A. Ability of lymph node and spleen cells to stimulate						
Experiment 1	LN	LN	870 ± 160	43,500 ± 4,900	42,600 ± 4,900	50 ± 11
	LN	Sp	2,550 ± 200	39,600 ± 1,000	37,000 ± 1,000	15.5 ± 1.3
	Sp	LN	4,720 ± 330	32,700 ± 2,500	28,000 ± 2,700	6.9 ± 0.8
	Sp	Sp	9,100 ± 800	25,070 ± 680	16,000 ± 1,000	2.7 ± 0.3
Experiment 2	LN	LN	2,280 ± 260	17,400 ± 1,100	15,400 ± 1,100	7.6 ± 1.0
	LN	Sp	3,440 ± 170	20,600 ± 1,200	17,200 ± 1,200	6.0 ± 0.5
	LN	Sp-FH	2,200 ± 200	12,500 ± 700	10,300 ± 700	5.6 ± 0.6
B. Ability of lymph node and spleen cells to respond						
Experiment 1	LN	LN	870 ± 160	43,500 ± 4,900	42,600 ± 4,900	50 ± 11
	Sp	LN	4,720 ± 330	32,700 ± 2,500	28,000 ± 2,700	6.9 ± 0.8
	LN	Sp	2,550 ± 200	39,600 ± 1,000	37,000 ± 1,000	15.5 ± 1.3
	Sp	Sp	9,100 ± 800	25,070 ± 680	16,000 ± 1,000	2.7 ± 0.3
Experiment 2	LN	LN	2,280 ± 260	17,400 ± 1,100	15,100 ± 1,100	7.6 ± 1.0
	Sp	LN	4,310 ± 120	11,600 ± 700	7,300 ± 700	2.7 ± 0.2
	Sp-FH	LN	6,060 ± 440	16,400 ± 900	10,300 ± 1,000	2.7 ± 0.2

In experiment 1 cell suspensions were prepared from mesenteric lymph nodes and spleens of CBA and (CBA x C57Bl)_{F₁} mice. Aliquots containing 1.0 million live cells and 1.0 million irradiated (2000R) cells in CMH/15% FCS were dispensed into microtest plates and labelled for 16 hrs after 72 hrs culture with H-3 thymidine. The second experiment was performed in an analogous way except that about 2 mls of spleen cell suspension (20-40 million cells) were layered on top of 2 mls of cold Ficoll-Hypaque and spun for 15 mins at 500g. White cells were then recovered from the interface, washed, and re-suspended in fresh medium. This procedure removed 95% of red cells; there was also about a 25% loss of white cells. Cultures labelled with H-3 thymidine after 76 hrs for 15 hrs. In the first experiment, figures for DNA synthesis are cp10m/100 microl. of culture, in the second experiment cp5m/100 microl.

cultures. Similar syngeneic effects have been found by other authors, principally in mixtures of thymus and spleen cells (401-405). Although in mixtures of neonatal thymus and adult spleen cells there is some reason to believe that the observed responses are real and may have an immunological significance (402-405), the apparent syngeneic responses obtained between cells from different organs of adult animals are most likely explained as culture artefacts. Thus, different cell populations may have varying abilities to provide feeder effects and thereby one cell population may be able to improve the survival of a second population. An alternative explanation is that mixing two populations of cells will change the proportions of the different types of lymphoid cell - T cells, B cells, macrophages, T cell subclasses. As a result, the likelihood of cooperative responses to components of the culture medium will be either increased or decreased according to the proportions of the cooperating cell types needed for optimal response. That the apparent syngeneic responses between lymph node and spleen cells observed here were caused by culture artefacts is shown by the finding that irradiated spleen cells increased the background transformation not only of live lymph node cells but also of live spleen cells. Such a finding is incompatible with a syngeneic MLR explanation. Furthermore, the apparent syngeneic stimulation of lymph node cells by irradiated spleen cells was abolished by spinning the spleen cells on Ficoll-Hypaque. Although it is possible that the 25% loss of white cells which occurred removed all cells having syngeneic spleen antigen a more likely explanation is that cells of a particular functional class were selectively depleted.

As far as responding ability is concerned the two experiments show that spleen cells are inferior to lymph node cells as judged by the stimulation in allogeneic cultures. Once again, because of the syngeneic effects described above, the index of transformation is distorted and not so reliable a guide as is the stimulation.

TABLE 19. Syngeneic effects in MLC.

	Control culture	"Mixed" culture	% Increase in DNA synthesis	
			Experiment 1	Experiment 2
A	Male v. Male _x	Male v. Female _x	31 ± 13**	- 1 ± 7 ^{NS}
	Female v. Female _x	Female v. Male _x	- 27 ± 6***	- 6 ± 5 ^{NS}
B	Young v. Young _x	Young v. Old _x	17 ± 11 ^{NS}	6 ± 6 ^{NS}
	Old v. Old _x	Old v. Young _x	- 2 ± 5 ^{NS}	4 ± 8 ^{NS}
C	LN v. LN _x	LN v. Sp _x		22 ± 6**
	Sp v. Sp _x	Sp v. LN _x		5 ± 3 ^{NS}

All cells used were from CBA mice, and except where stated otherwise (individual sections A, B, C) were obtained from the mesenteric lymph nodes of 8 week old male mice. In each section one of the quantities sex, age, or organ or origin were disparate, the other two quantities were identical. The old CBA mice used were 12 months old. All cultures were at a 2:1 ratio of responder:stimulator cells, the latter having received 2000R. All cultures labelled after 72 hrs for 15-17 hrs. The levels of significance of the responses between disparate cells are shown.

Ficoll-Hypaque treatment was used in the second experiment to ascertain whether red cells present in spleen cell suspensions could be inhibiting MLC responses as found in Chapter 4 Section 1. The results suggest that red cells were at best playing only a small part in the reduced responsiveness of spleen cell populations in MLC. Surprisingly, Ficoll-Hypaque treated spleen cells had reduced stimulatory powers in MLC. Since red cells do not stimulate in MLC (15, 358,406) the result serves to emphasise the point that Ficoll-Hypaque treatment can have effects on cell populations in addition to the simple removal of red cells.

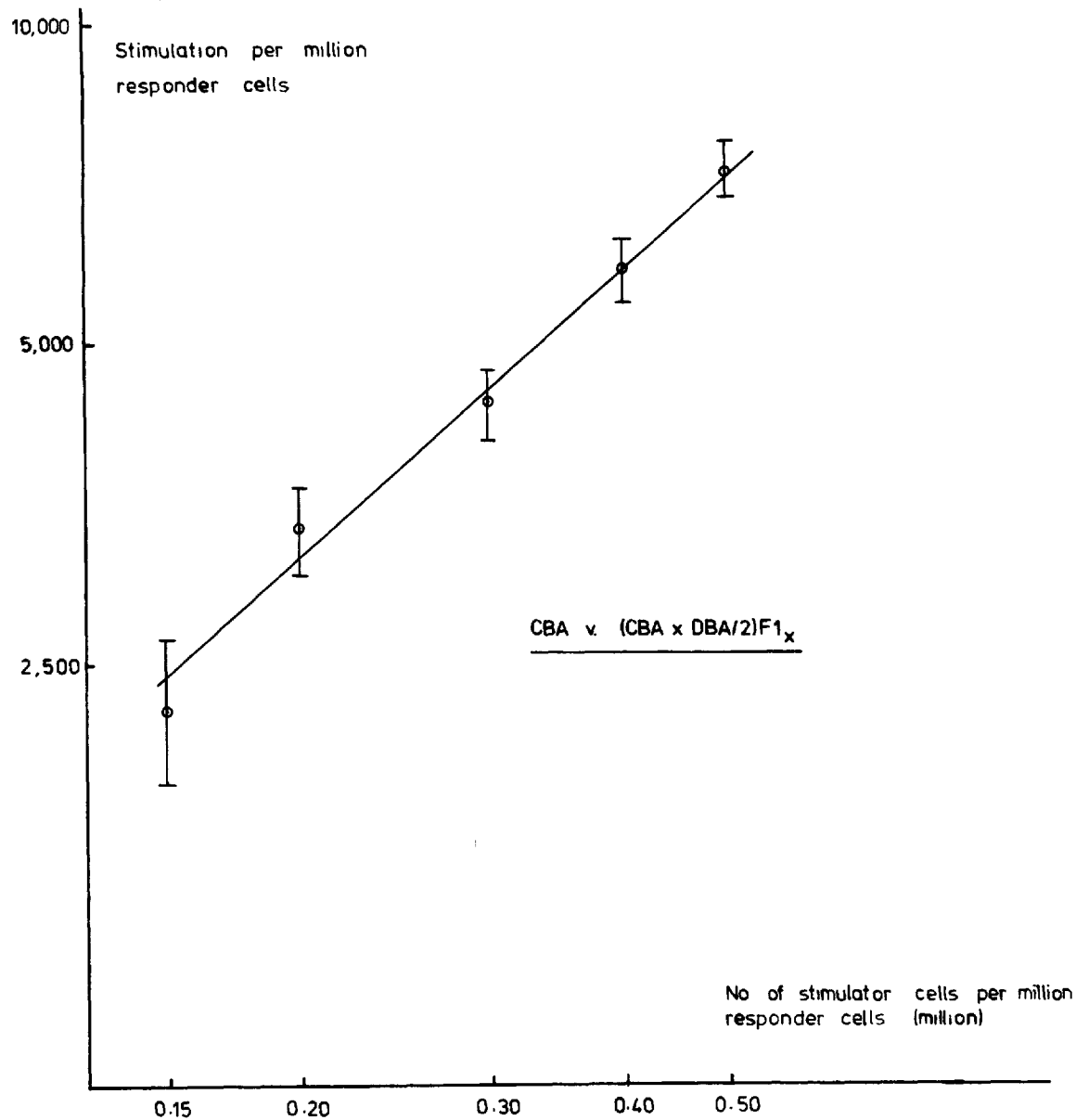
In virtually all MLC experiments described in this thesis lymph node cells were used as responder cells. In most cases lymph node cells were also used as stimulator cells, but in a few experiments spleen cells were used.

(b) Syngeneic effects.

To examine whether syngeneic effects could be found in other situations than that described above, the experiments in Table 19 were performed.

Once again lymph node cells showed a significantly higher background level of DNA synthesis in the presence of irradiated syngeneic spleen cells than in the presence of syngeneic lymph node cells. No other significant syngeneic effects were found except that in one experiment male lymph node cells showed an elevated level of DNA synthesis in the presence of irradiated syngeneic female cells compared to cultures with irradiated male cells. That this effect was of non-immunological origin was shown by the fact that female cells also made more DNA in the presence of female than in the presence of male cells. In the second experiment this effect of irradiated female cells could not be detected.

FIGURE 5. Kinetics of MLC responses with irradiated F1
stimulator cells.



10. Kinetics of MLC reactions.

(a) Dose-response curves.

Having established conditions under which mouse MLC reactions could be easily and accurately measured it was important to show that increasing amounts of antigen in culture would give a continuously increasing MLC response. This was demonstrated using both live and irradiated stimulator cells (Figures 5 and 6), with the following protocols.

Two experiments were performed with live stimulator cells. Varying numbers of CBA and (CBA x A) F_1 lymph node cells were mixed together such that the total cell number in each well of a microtest plate was a constant 1.0 million. In the first experiment cells were cultured in CMH/10% FCS, in the second experiment in CMH/15% FCS. Cultures were incubated at 33-34° for 72 hrs and then labelled for 16 hrs with H-3 thymidine. The results obtained in the two experiments did not differ significantly from each other. Therefore, the mean values were calculated and these are shown in Figure 6.

In the experiment with irradiated stimulator cells, each well contained 1.00 million CBA lymph node cells mixed with varying numbers of irradiated (2000R) (CBA x DBA/2) F_1 lymph node cells together with a complementary number of irradiated (2000R) CBA lymph node cells such that at all responder:stimulator cell ratios there were 1.00 million live CBA cells and 0.50 million irradiated cells. The cells were cultured in CMH/20% FCS for 72 hrs at 33-34° before labelling with H-3 thymidine for 22 hrs.

The results (Figure 5) show that when the logarithm of the stimulation in mixed cultures is plotted against logarithm of antigen concentration the data fit a straight line remarkably well over the range of antigen concentrations tested. But, an interesting difference exists between the response obtained against live F_1

stimulator cells and that obtained against irradiated F_1 cells. With the latter stimulator cells the slope of the log v. log dose-response curve is very close to unity, i.e. the straight line.

$$\log S = k \log [Ag]_{Irr} + c \quad (1)$$

becomes

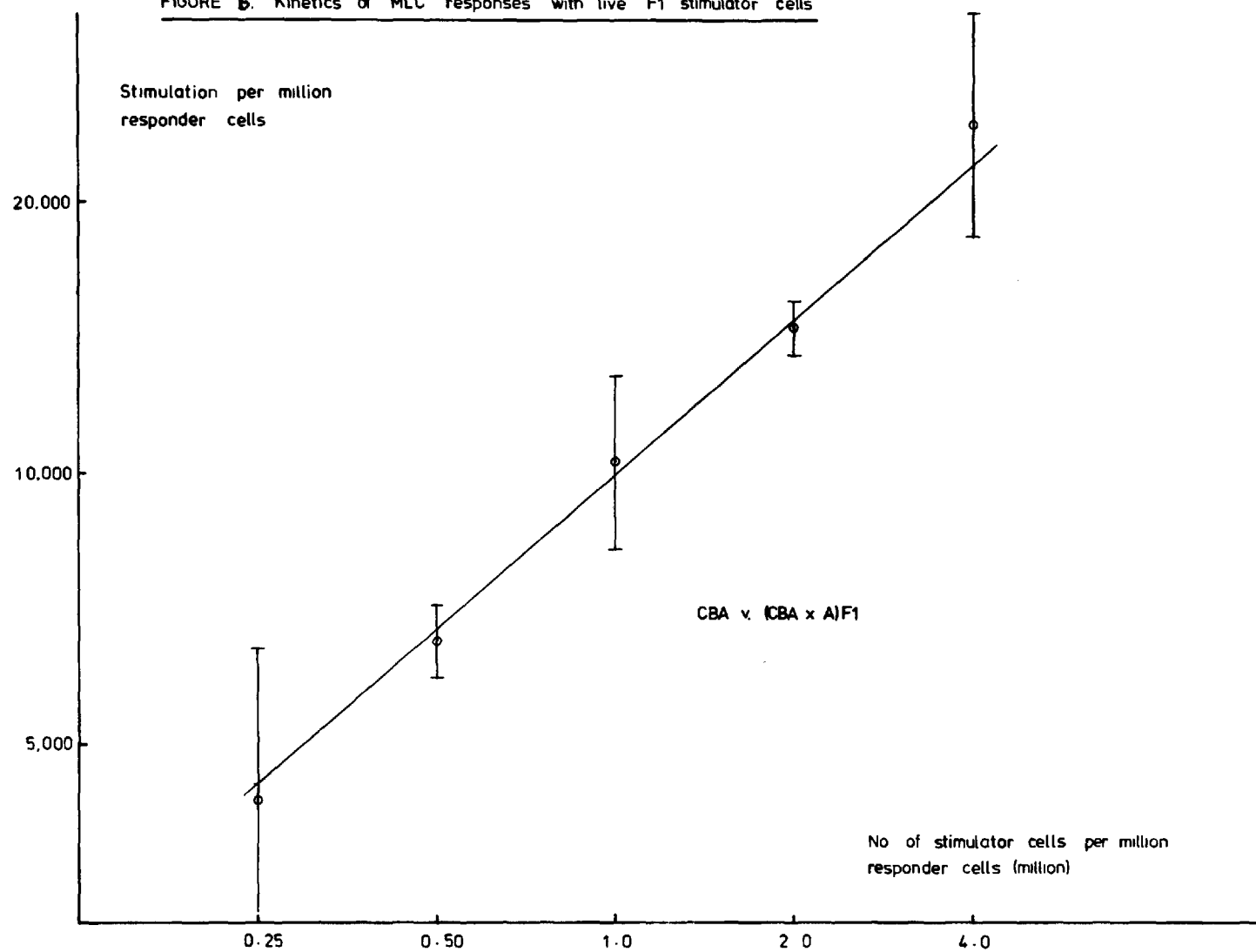
$$\log S = \log [Ag]_{Irr} + c \quad (2)$$

$$S = m [Ag]_{Irr} \quad (3)$$

where S is the stimulation obtained in mixed cultures with an antigen concentration $[Ag]_{Irr}$, and where c and m are constants such that $c = \log m$. Equation 3 shows that a plot of S v. Ag_{Irr} , i.e. a double arithmetic plot, will also be linear, passing through the origin with a slope of m . This means that each stimulatory cell induces a reaction in the responding cell population which is independent of the presence of other stimulating cells. Thus over the range of stimulating cell concentrations studied each one is recognised as a distinct and separate entity. This apparent lack of competition between stimulating cells suggests that their numbers are small compared to the numbers of cells in the responding population which have receptors directed against them. Because relatively large numbers of irradiated F_1 cells were added to the mixed cultures (10:1 to 2:1 responder:stimulator cell ratios) the finding can be explained in one of two ways (which are not mutually exclusive):

- (i) irradiated stimulator cells die rapidly in culture, so that either all stimulator cells possess MLC Ag's on their surface for only a short time, or that only a small proportion of cells survive long enough for an MLC response to be generated against them. The decreased stimulatory ability of cell populations given increasing doses of radiation (see Section 7b) supports the finding (398) that irradiated cells rapidly degenerate in culture to a non-immunogenic form.

FIGURE 6. Kinetics of MLC responses with live F1 stimulator cells



- (ii) MLC antigens are present on only a small subpopulation of lymphocytes. It is already known that the major part of MLC stimulatory activity resides on B cells (see introduction to this chapter). Since only about 20% of mouse lymph node cells are B cells this could result in only one-fifth of the cells in a stimulating population being capable of stimulation, even assuming that all B cells are equally effective. Furthermore, it has been postulated that LD antigens may be clonally distributed (407,408) so that only a small proportion of cells carry a given MLC antigen.

For these explanations to be acceptable one must also assume that there are a relatively high number of MLC antigen-reactive cells in the responder population. There is evidence that this is the case, the percentage of cells responding in MLC and GVH reactions against major histocompatibility locus differences being of the order of 1-12%, at least in the rat (409).

The kinetics of stimulation by live cells are somewhat different: the gradient of the log v. log plot is approximately 0.6. Consequently the corresponding equations 2 and 3 become

$$\log S = 0.6 \log [Ag]_c + c'$$

$$S = m' \sqrt{[Ag]_c}$$

The double arithmetic dose-response plot using live stimulator cells is thus a paraboloid passing through the origin. This means that the ability of a given cell to stimulate cells in the responding population decreases progressively as the total number of stimulator cells increases. The different kinetics shown by MLCs

TABLE 20. Attempts to detect gene-dosage effects in mouse MLC.

Experiment number	Stimulation measured in cultures:		Ratio of fully allogeneic:semiallogeneic	Significance of difference from 1.0	Significance of difference from 2.0
	<u>CBA v. (CBA x A)₁F₁</u>	<u>CBA v. A_x</u>			
1	4,380 ± 780	3,180 ± 330	0.73 ± 0.15	NS	***
2	6,600 ± 800	20,400 ± 1,600	3.24 ± 0.47	***	-
	<u>CBA v. (CBA x DBA/2)₁F₁</u>	<u>CBA v. DBA/2_x</u>			
1	12,300 ± 670	19,800 ± 1,200	1.61 ± 0.13	***	*
2	75,300 ± 3,300	132,600 ± 2,100	1.76 ± 0.08	***	***
3	23,990 ± 380	27,590 ± 520	1.15 ± 0.03	***	***
	<u>CBA v. (CBA x C57B1)₁F₁</u>	<u>CBA v. C57B1_x</u>			
4	42,100 ± 4,700	31,700 ± 3,850	0.75 ± 0.12	NS	***
	Mean		1.34 ± 0.31	NS	NS

Aliquots of 1.0 million (experiments 1, 2 and 3) or 1.2 million (experiment 4) CBA lymph node cells were cultured with 0.50 million irradiated (2000R) lymph node (experiments 1 and 2) or spleen (experiments 3 and 4) cells of the hybrid and parental strains shown. Cultures were labelled with H-3 thymidine after 72 (experiments 1, 2 and 3) or 96 (experiment 4) hrs for 16-18 hrs. Hybrid and parental cell donors were always of the same age and sex.

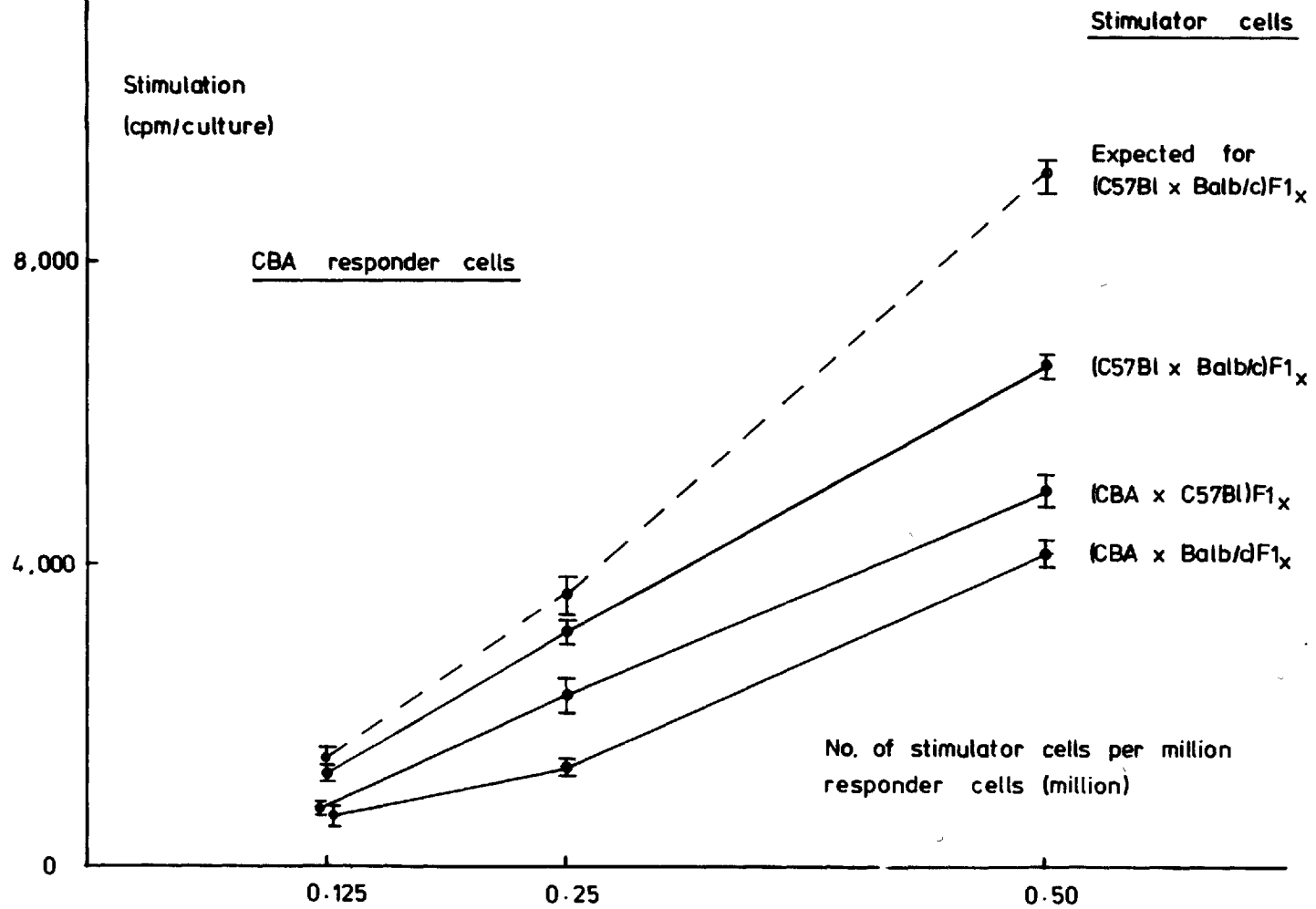
with live cells suggests that the linear arithmetic kinetics shown by cultures with irradiated cells are due to radiation damage of the stimulating population. However, since the experiment with live cells was performed over a slightly different range of antigen concentrations and with a different strain of antigen further investigation would be required to confirm the different kinetics.

(b) Gene dosage effects.

Gene dosage effects in mixed lymphocyte cultures have now been detected in many systems including humans (410,411), pigs (412), rats (413) and mice (414). The magnitude of the gene dosage effect is variable. In an exhaustive study of human siblings differing by 0, 1, or 2 haplotypes Dupont et al (411) found that in cultures of cells differing by 2 haplotypes the degree of stimulation was almost exactly twice that found in cultures where the cells differed by only 1 haplotype. At the other extreme, Wilson and Nowell's studies of rat MLC (415) showed no gene dosage effect at all, a finding consistent with their apparent use of saturating amounts of antigen.

Table 20 shows the results of a number of experiments using three different strain combinations. In 2/6 cases no gene dosage effect was detectable, and in 3 of the remaining 4 cases the response with fully allogeneic stimulator cells was still significantly less than twice the response obtained with F_1 stimulator cells. Since transplantation antigens behave as the products of co-dominant alleles one would expect that fully allogeneic cells would have twice as much LD alloantigen on their cell surface as do semi-allogeneic cells. The kinetics of MLC reactions in this system with irradiated stimulator cells are linear on a double-arithmetic plot (Section 10a). Thus, doubling the number of

FIGURE 7. MLC responses to various F1 stimulator cells



stimulator cells causes double the stimulation in mixed cultures. Yet, in the experiments shown in Table 20 allogeneic cells did not cause 2.0 times the stimulation of semi-allogeneic cells. Three explanations are possible:

- (i) There is a possibility that some experiments in Table 20 did not follow linear arithmetic kinetics.
- (ii) Allogeneic cells do not have twice the amount of LD antigen as found on semi-allogeneic cells due to feedback inhibition of the synthesis of these particular proteins in parental cells.
- (iii) Two antigens on the same cell do not stimulate as much as when they are on separate cells.

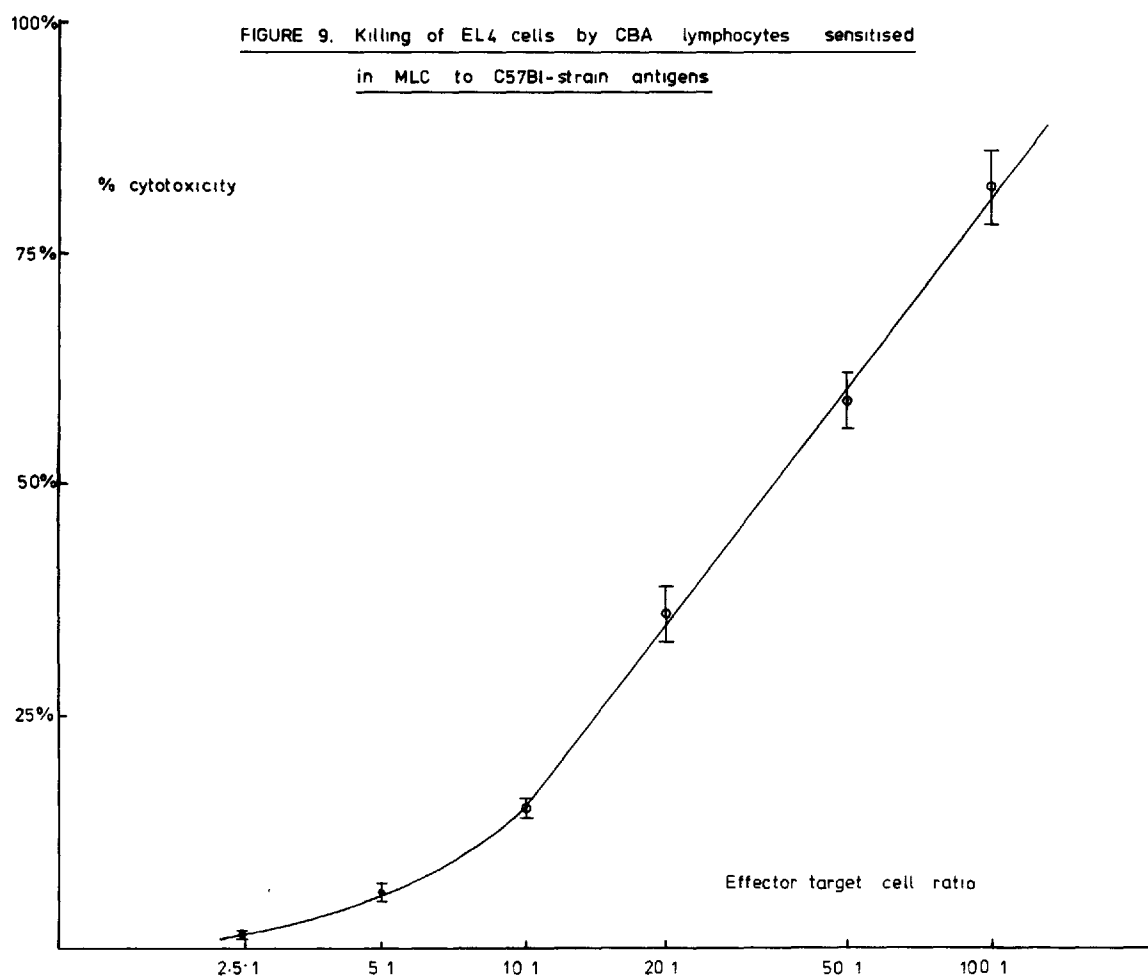
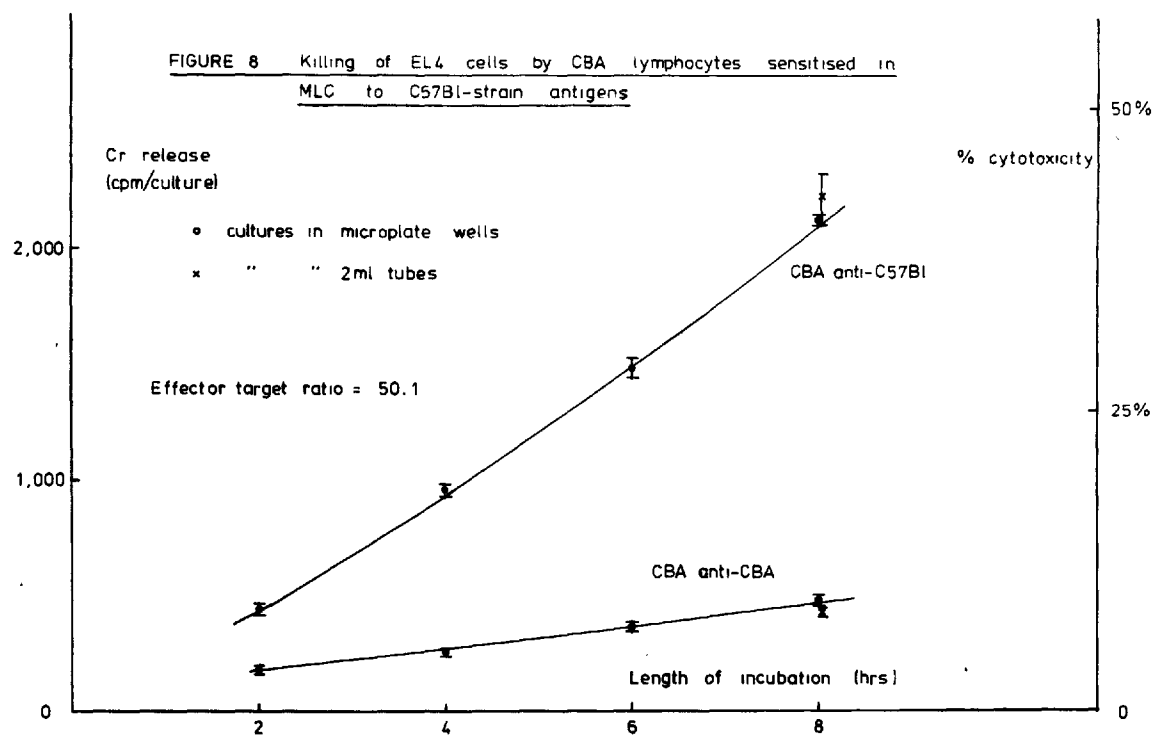
Some indication as to which one of these explanations is correct is provided by the experiment shown in Figure 7. 1.0 million CBA lymph node cells were cultured with varying numbers of (CBA x Balb/c) F_1 , (CBA x C57B1) F_1 , or (C57B1 x Balb/c) F_1 lymph node cells in CMH/10% FCS. Stimulator cells were from pools of three age-matched female mice and all were given 2000R of X-irradiation. Cultures were labelled with H-3 thymidine after 72 hrs for a further 18 hrs.

Figure 7 shows that the kinetics conform closely to linearity on a double arithmetic plot and are therefore additive arithmetically. The responses of CBA cells to irradiated (CBA x Balb/c) F_1 and to irradiated (CBA x C57B1) F_1 cells were similar. These two responses were added together to give the response expected against irradiated (C57B1 x Balb/c) F_1 cells. As can be seen the observed response was considerably lower than expected. Since the fully allogeneic cells possessed two different strains of antigen, explanation (ii) above cannot be applicable to this experiment. Explanation (i) has already been ruled out since the kinetics in this experiment were confirmed to be very close to linear. Thus, it seems that when two antigens are on the same cell they are less effective stimulants in MLC than when they

are on different cells. This explains the relatively low gene dosage effect.

The implication of this finding is that there is competition in mixed lymphocyte cultures amongst receptor-bearing responder cells for the antigen on the surface of a given cell; when one cell has recognised the foreignness of a particular cell the chances of another cell recognising the same stimulator cell are reduced. The most obvious way that this could happen is through steric effects. That steric effects are important in mixed lymphocyte interactions is shown by the work of McFarland and Heilman (416) using time-lapse cinematography of mixed cultures. By day 2 in mixed cultures but not in control cultures lymphocytes were seen to aggregate around macrophages and by the third day transformation of some of these cells was observed. Thus, prolonged attachment of lymphocytes to macrophages occurs during the induction of MIC response. It may well be that a necessary pre-condition for responder cells to react against stimulator cells is that both cells become temporarily attached and immobilised on the surface of a macrophage.

An alternative explanation is that the antigen on irradiated cells has a short half-life (398; see also above discussion of kinetics) and therefore each stimulator cell will have a diminished probability of interacting with a second responder-strain cell. This hypothesis seems more likely than that of competition on macrophage surfaces since Wilson and Nowell (415) found that in an experiment of similar design the responses in A v. (B x C) F_1 cultures were close to the values expected from summation of the responses found in A v. (A x B) F_1 and A v. (A x C) F_1 cultures. The principal difference between their experiments and the ones described here was that they achieved one-way responses in A v. (B x C) F_1 by using (B x C) F_1 cells from animals made tolerant to A, i.e., their stimulator cells were live cells.



11. Generation of cytotoxic cells in MLC reactions.

It is now well established that cytotoxic cells are generated during MLC reactions (339). To check that cytotoxic cells were produced in the MLC system described above, CBA lymph node cells were cultured either alone (CBA anti-CBA) or mixed in a 1:1 ratio with live (CBA x C57B1) F_1 cells. After $4\frac{1}{2}$ days culture at $33-34^{\circ}$ in a microtest plate the cells were gently resuspended, washed, and adjusted to 5 million viable cells/ml in CMH/20% FCS. They were mixed with an equal volume of Cr^{51} -labelled EL4 cells to give an effector:target cell ratio of 50:1 and 0.2 ml aliquots (containing a total of 0.5 million viable cells) were dispensed into either microtest plates or 2 ml plastic tubes. The cultures were then incubated at $33\frac{1}{2}^{\circ}$ for various times. The results shown in Figure 8 clearly demonstrate the presence of cytotoxic cells after mixed lymphocyte culture. After the first 2 hrs the cytotoxic reaction proceeds in an approximately linear manner with time. Figure 8 also shows that there was no significant difference between the rates of the cytotoxic reaction in microtest plates and in tubes. Therefore, for the sake of convenience, the former were used for all cytotoxic reactions described in this thesis.

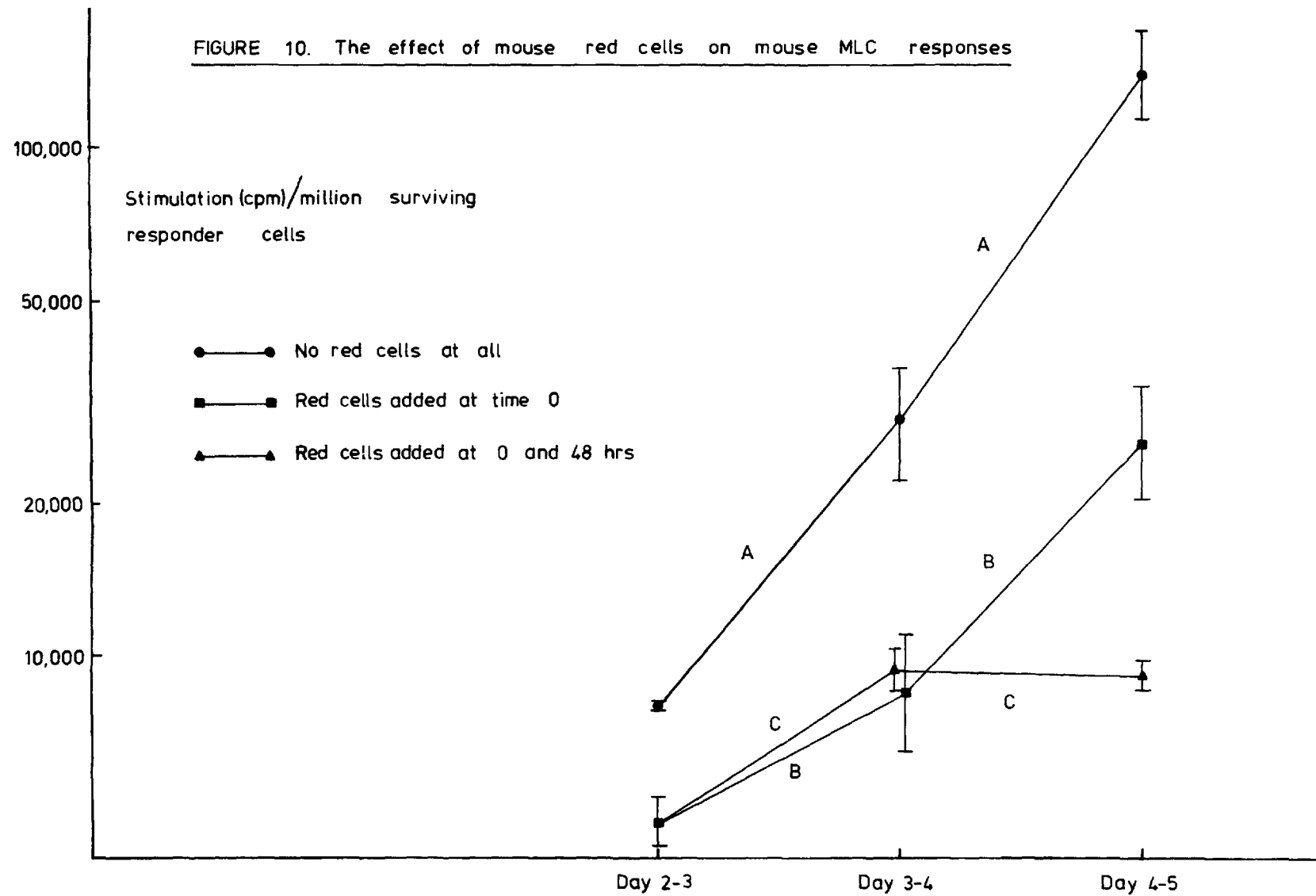
It has also been shown that the release of Cr from target cells in the Cr-release assay for cytotoxic cells increases linearly with the logarithm of the ratio of effector:target cells (17,238). To confirm that normal kinetics were being followed in the microtest plate system the dose-response curve was studied. CBA lymph node cells were cultured with irradiated CBA and C57B1 spleen cells for $4\frac{1}{2}$ days. They were then washed, resuspended in fresh medium, and various dilutions made. The dilutions of effector cells were mixed with a constant number of Cr^{51} -labelled EL4 cells to give effector:target cell ratios from 2.5:1 to 100:1. Cultures were incubated for

6½ hrs at 37°. The result is shown in Figure 9. Linear kinetics were obtained at cell ratios giving between 15% and at least 80% killing. Two further small pieces of information emerged from this experiment (data not shown). The spontaneous Cr-release rate from target cells increased slightly but continuously as the effector:target cell ratio increased; and the maximum chromium release from target cells frozen and thawed 3 times decreased slightly as the effector:target cell ratio increased. Thus, in all cytotoxic experiments described below target cells were not only cultured with cells sensitised against allogeneic cells but also with cells "sensitised" against syngeneic cells, and at the same "effector":target cell ratio. Also, wherever possible, maximum Cr-release was ascertained by freezing and thawing target cells in the presence of the appropriate number of effector cells.

12. Conclusion.

In this chapter an investigation of various aspects of the mouse MLC technique devised by Festenstein has been described. Its adaptation to microtest plates was shown to be feasible and the greatly improved cell survival and precision of assay of MLC reactivity under conditions of monolayer formation and low incubation temperature were shown to lead to linear dose-response kinetics. The generation of cytotoxic cells during MLC was shown and the classical reaction kinetics of these against target cells was demonstrated in a Cr-release assay adapted to microtest plates. This MLC system was used for an investigation of immunological unresponsiveness in the mouse described in Chapters 5 and 6. During the preliminary study of MLC technique a number of unexpected observations were made and a description of a brief investigation of some of these findings is given in the next chapter.

FIGURE 10. The effect of mouse red cells on mouse MLC responses



Factors causing inhibition or enhancement of mouse MLC responses.1. Effect of red blood cells on lymphocytes in culture.

In the description of the mouse MLC technique by Festenstein et al red blood cells syngeneic to the responder strain were added to cultures at a rate of 20 microl. of packed red cells/10 mls of culture suspension (317,417). After performing a number of experiments in this way it was decided to investigate what effect they were having on MLC responses in the hope that it would be possible to perform cultures without them, thus avoiding some possible difficulties in the interpretation of results and the extra labour involved in adding them.

Figure 10 shows the results of a time course experiment in which CBA, (CBA x A) F_1 , and 1:1 mixed lymph node cell suspensions were each divided into two parts, to one of which CBA whole blood cells were added at a concentration of 20 microl. packed cells/10 mls of culture. At this dilution of whole blood cells there are about 20 million red cells/ml and 0.08 million white blood cells/ml. Since the lymph node cell suspensions were at 2.50 million/ml the extra amount of white cells added is negligible and the final ratio of lymphocytes to red cells is about 1:12. 0.5 ml aliquots of both types of culture were dispensed into tubes. Cultures with no red cells were refed after 48 and 88 hrs with 0.5 mls of plain fresh medium. Cultures containing red cells were either refed at 48 and 88 hrs with plain medium or at 48 hrs with medium containing 20 microl. packed fresh CBA red cells/10 mls and at 88 hrs with plain medium. Cultures of each group were labelled after 48, 72 and 96 hrs with H-3 thymidine for 16 hrs. The stimulation obtained in mixed cultures on each day was divided by the CBA cell survival at the end of the labelling period to give an estimate of the stimulation per million surviving responder cells. The reason for adding extra red cells to some cultures after 48 hrs is that by this

TABLE 21. Effect of mouse red cells on mouse lymphocytes in vitro.

Initial ratio of red cells:lymphocytes	Strain of red cell added	Background in unmixed cultures ⁺⁺		Response in mixed cultures		Stimulation in PHA cultures ⁺	Inhibition of stimulation in:			% Survival of CBA cells
		CBA	(CBA x C57B1)F ₁	Stimulation ⁺⁺	Index		CBA background	NIC	PHA	
0	CBA ⁺ (CBA x C57B1)F ₁ ⁺	2,570 ± 40 2,300 ± 220	350 ± 40	9,400 ± 680	7.5 ± 0.6	30,600 ± 1,500	-	-	-	16 ± 2
1 : 1	CBA (CBA x C57B1)F ₁	1,390 ± 110 1,760 ± 140	280 ± 40	6,210 ± 350	8.4 ± 0.7	25,900 ± 1,600	46	34	15	20 ± 2
2 : 1	CBA (CBA x C57B1)F ₁	1,300 ± 90 1,410 ± 50	160 ± 30	3,080 ± 90	5.2 ± 0.4	13,100 ± 1,800	49	70	57	15 ± 2
5 : 1	CBA (CBA x C57B1)F ₁	1,340 ± 70 1,510 ± 20	320 ± 40	2,430 ± 300	3.9 ± 0.4	7,700 ± 1,200	48	74	75	17 ± 2
10 : 1	CBA (CBA x C57B1)F ₁	1,100 ± 60 1,180 ± 50	190 ± 30	1,630 ± 120	3.5 ± 0.2	6,300 ± 800	57	83	79	19 ± 2
20 : 1	CBA (CBA x C57B1)F ₁	720 ± 50 760 ± 40	270 ± 40	1,170 ± 230	3.3 ± 0.5	9,900 ± 800	72	88	67	21 ± 2

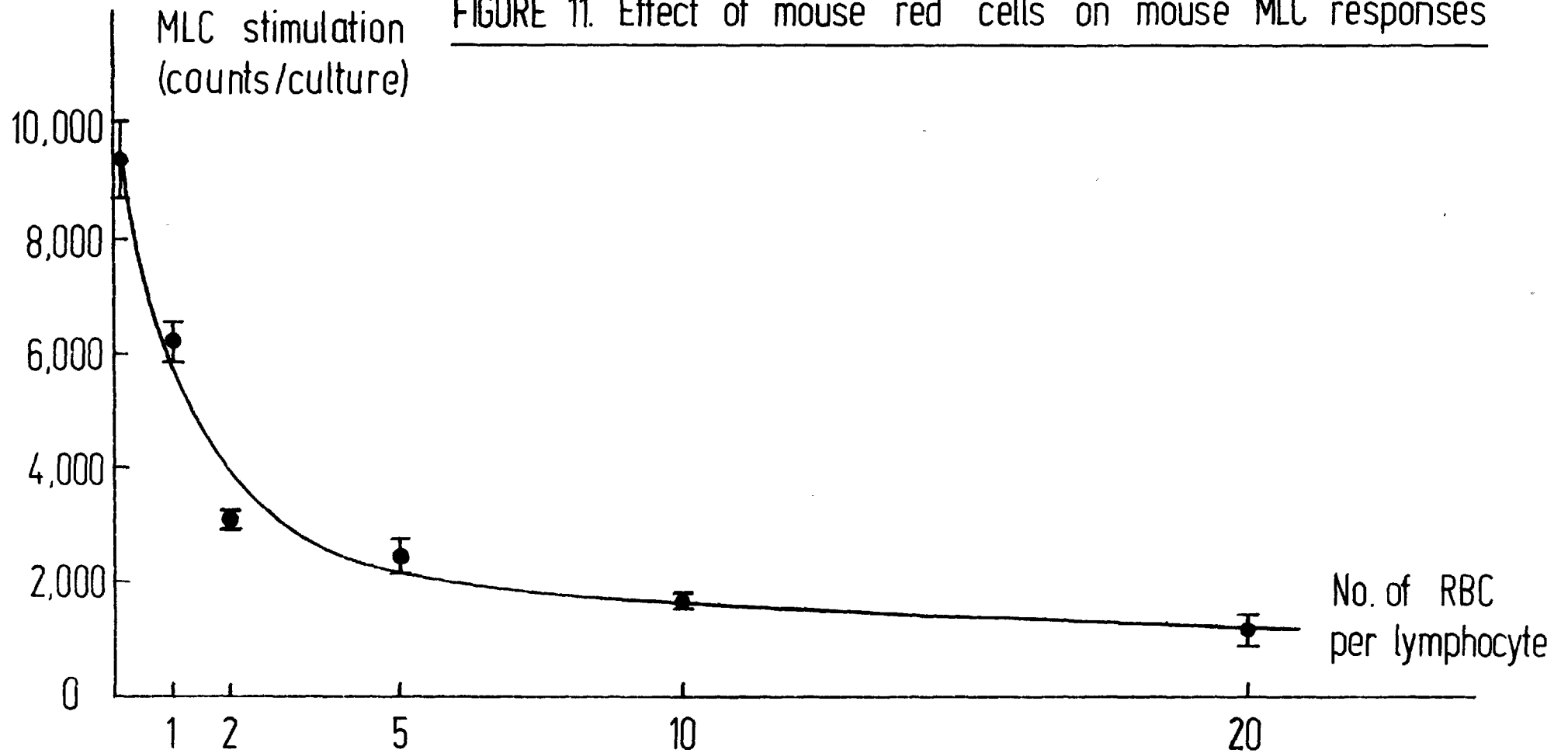
Portions of CBA and (CBA x C57B1)F₁ lymph node cells were mixed with varying numbers of twice-washed ** CBA whole blood cells to give suspensions with the final red cell:lymphocyte ratios shown. 1:1 mixed cultures were formed and aliquots of 1.0 million control and mixed cells were dispensed into wells of a microtest plate. To other portions of CBA lymph node cells varying numbers of twice-washed (CBA x C57B1)F₁ whole blood cells were added and aliquots of 1.0 million cells dispensed into the same microtest plate. Finally, to further portions of CBA lymph node cells varying numbers of CBA whole blood cells were added followed by reconstituted PHA to give final PHA concentration of 1/65. Aliquots of 1.0 million cells from these suspensions were dispensed into a second microtest plate. All cultures were incubated for 77 hrs before labelling with H₃-thymidine for 16 hrs.

+ Supernatant from CBA or (CBA x C57B1)F₁ red cell suspensions was added.

++ cpm/100 microl. of culture

** Citrated blood was washed twice in this experiment to exclude all possibility of citrate being carried over into culture: this possibility was further controlled for by adding supernatants from washed red cells to cultures (see footnote⁺).

FIGURE 11. Effect of mouse red cells on mouse MLC responses

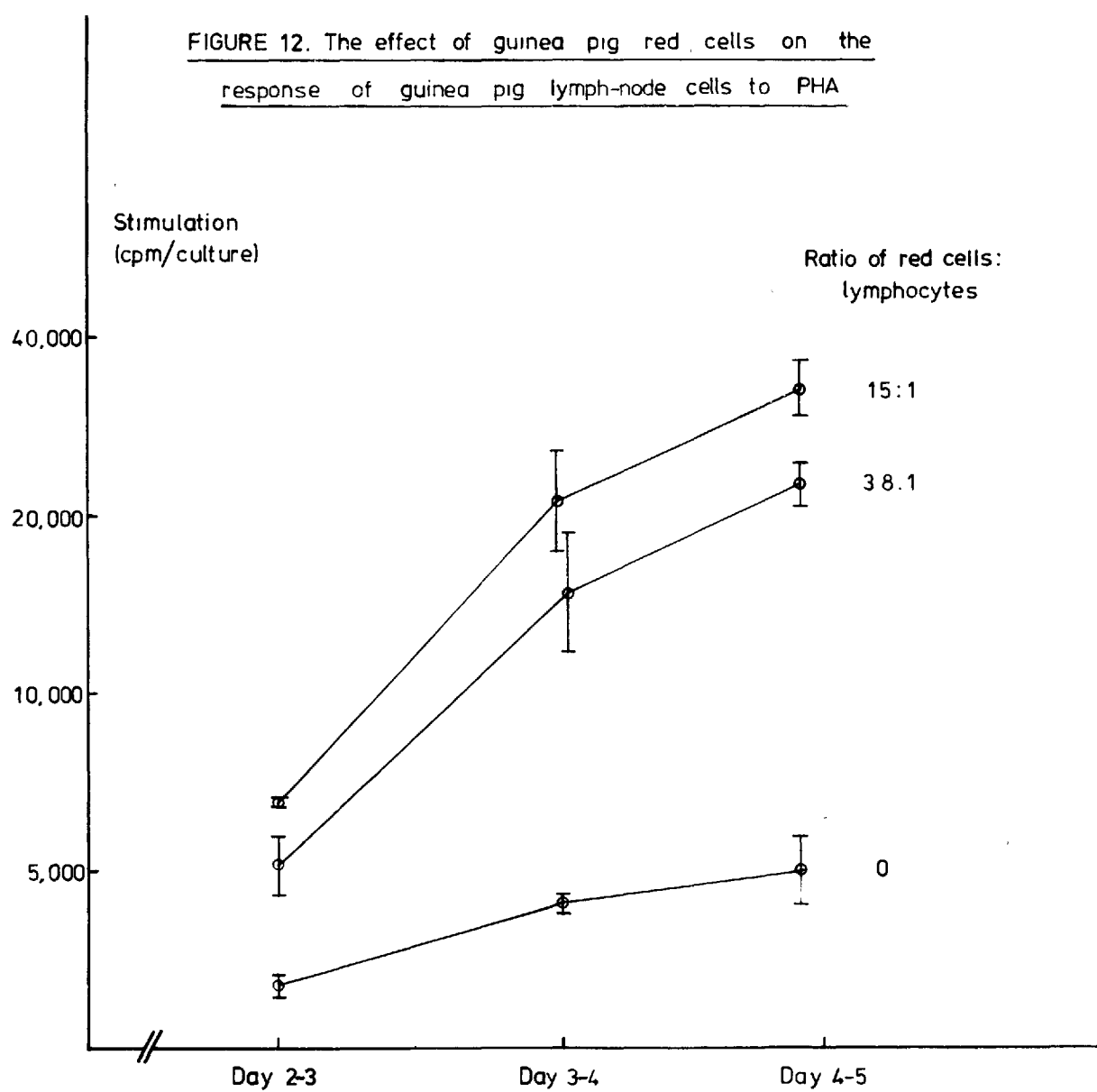


time most of the red cells added at the beginning of culture have lysed and refeeding with red cells at this time maintains the presence of intact red cells in culture for up to 96 hrs.

The result of this experiment was quite unexpected. The red cells caused a strong inhibition of MLC response. To investigate this further a detailed dose-response experiment was done. The results of this are shown in Table 21 and Figure 11. Thymidine uptake in unstimulated, PHA-stimulated, and MLC-stimulated CBA lymph node cells was progressively inhibited by increasing amounts of CBA red cells. (CBA x C57Bl) F_1 red cells had a similar effect on unstimulated CBA lymphocytes, although at some dosages there is the suggestion of a trace of MLC stimulatory activity in the F_1 red cells, presumably from the small numbers of white cells present (see description of time-course experiment above). Despite the inhibitory effect of mouse red cells there seems to be no loss of viability in the CBA cells so that the inhibition of thymidine uptake by lymphocytes is not caused by general toxic properties of red cells in culture (see below for such effects of normal mouse serum) or by a suffocation of lymphocytes by the excess of red cells. Indeed, in some experiments (not shown) mouse red cells have been found to enhance the survival of mouse lymphocytes in culture.

Valuable information as to the mechanism of inhibition came from an experiment in which the effect of guinea-pig red cells on the response of guinea-pig lymph node cells to PHA was measured (Figure 12). Portions of a Heston strain guinea-pig lymph node cell suspension were mixed with different amounts of a suspension of twice-washed Heston whole blood cells to give final lymphocyte concentrations of 2.0 million/ml and red cell concentrations of 0, 7.5, or 30 million/ml. To half of each suspension PHA was added to a final concentration of 1/170 and 0.50 ml aliquots of control and PHA suspensions dispensed into tubes. Cultures were refed with 0.50 mls of medium after 50 and

FIGURE 12. The effect of guinea pig red cells on the response of guinea pig lymph-node cells to PHA



90 hrs culture and labelled with C-14 thymidine after 52 (these cultures were not refed), 75 and 99 hrs for 16 hrs.

In contrast to the situation in the mouse, the response of guinea-pig lymph node cells to PHA is strongly enhanced by the presence of syngenic red cells. Similar results were found in a second experiment. The most striking difference between the behaviour of guinea-pig red cells and mouse red cells is that the former survive intact throughout the whole of the culture period whereas the latter have virtually all lysed within 48 hrs. The implication is that it is the disintegration of mouse red cells which inhibits the PHA response.

The ability of red blood cells to enhance PHA responses is an interesting phenomenon and has previously been reported to occur in human lymphocyte cultures. Both Yachnin et al (418) and Johnson et al (419) found that the mitogenic properties of erythro-agglutinating components of PHA preparations could be increased if red cells were added to the cultures. Yachin et al found that red cell:lymphocyte ratios as low as 1:1 caused considerable improvement of the PHA response, and furthermore that both red cell stroma and even heavily irradiated lymphocytes were able to cause enhanced responses. A rationalisation of these experiments was provided by Greaves and Bauminger (96) who reported that when PHA was covalently bound onto Sepharose beads it acquired the ability to stimulate B cells, presumably due to its being presented to lymphocytes as a series of repeating determinants. It therefore seems likely that part of the enhancement of PHA responses caused by adding red cells is due to the ability of surface-bound PHA to stimulate B cells so that both B and T cell responses will occur in cultures containing red cells. Other factors are presumably operating, however, since the magnitude of the enhancement of PHA responses by red cells is much greater than can

be accounted for by the likely ratio of B and T cells in guinea-pig lymph node or human peripheral blood cells. It appears that addition of red cells must also increase the efficiency with which PHA stimulates T cells.

Eijsvoogel and colleagues (391) also found that red cells enhanced the response of human lymphocytes to PHA, but by contrast MLC responses and responses to soluble antigens were inhibited by red cells. It therefore seems that enhancing effects by red cells are limited (a) to mitogens and (b) to species of animals whose red cells do not lyse significantly during culture periods. Since red cell stroma prepared by osmotic lysis retains the enhancing properties (418) it may well be that the mouse red cells, although able to bind PHA rapidly undergo degenerative membrane changes which prevent the bound PHA from being able to stimulate lymphocytes.

The inhibition of MLC by red cells is presumably not primarily due to disintegration of the red cells since the same effects are found in human cultures (391) where red cells survive well. Since MLC and antigen-induced in vitro responses appear to have a more stringent requirement for macrophages than do mitogen-induced responses (420 - 423) it may be that the inhibition of the former responses is caused by some interference by red cells with macrophage function. One possibility is that if the binding of lymphocytes and/or antigen to the surfaces of macrophages is a necessary pre-requisite for a response to occur, as implied by the studies of McFarland and Heilman (416), then red cells could sterically inhibit this process. That red cells do not act by a direct steric hinderance of the interaction between histo-incompatible lymphocytes is implied by the observation that red cells also inhibit the background transformation of lymphocytes. Such a hypothesis is consistent with the delayed effect of the

inhibition caused by adding red cells. (Figure 10): when extra red cells were added to some cultures on curve B at 48 hrs no effect was noticeable until 48 hrs after their addition. However, various other mechanisms by which red cells may inhibit in vitro responses can be envisaged, and further investigation of the phenomenon would be desirable in view of the recent publication of methods for whole blood culture (424-425). Although apparently good responses have been found in such cultures to mitogens, antigens, and allogeneic cells caution will be needed in the interpretation of results obtained in such systems until detailed comparative studies of the responses in whole blood and purified lymphocyte cultures can be made.

2. The effect of normal mouse serum on mouse lymphocytes in culture.

It is a rather remarkable fact that mouse serum should be toxic to mouse lymphocytes. But as the results obtained in the experiments described below show, any concentration of mouse serum greater than 10% is extremely toxic, there being a measurable decrease in the viability of cultured cells at concentrations as low as 1%, and an inhibition of DNA synthesis at concentrations much lower than this. Usually serum was used unheated, but as can be seen in Table 22 heated serum is also very toxic to cultured cells. Thus the toxicity exists

TABLE 22.

Toxic effects of normal mouse serum.

Experiment	Strain of lymphocyte	Mouse serum concentration		
		1%	2%	5%
1	CBA	ND	67 $\pm$ 6	61 $\pm$ 6
2	CBA	96 $\pm$ 13	86 $\pm$ 10	73 $\pm$ 8
1	(CBA x A) F_1	ND	91 $\pm$ 6	54 $\pm$ 5
2	(CBA x A) F_1	ND	ND	71 $\pm$ 6

CBA and (CBA x A) F_1 lymph node cells were cultured in CM/10% FCS (batch 40367) in tubes in the presence the amounts of mouse serum shown. In both experiments mouse serum was obtained by syringe bleeding

of 6 three-month old female CBA mice given nembutal anaesthetisation (see Materials and Methods). Sera were heated at 56° for 30 mins. and filtered through a sterilising Millipore membrane. After 90 hrs culture at $33\frac{1}{2}^{\circ}$ the cells were fixed with glutaraldehyde and viable cell counts performed after coding and randomisation of tubes. Figures are the percentage survivals relative to cultures with no mouse serum $\pm$ the standard error estimated from the variance between replicate tubes.

in the absence of active complement and, as shown in the table, is equally potent against both syngenic and semi-allogenic cells. These observations show that antibody cannot be involved. The toxic effects of mouse serum are even more dramatic when the DNA synthesis of lymphocytes cultured in the presence of mouse serum is measured, Table 23. With only 1% mouse serum the DNA synthesis in unmixed cultures was reduced by about 70% compared to cultures with no mouse serum, and with 10% mouse serum there was no detectable DNA synthesis at all. There was no significant difference between the inhibition in (CBA x A) F_1 cells caused by mouse serum. The effects caused by adding mouse serum to cultures were in complete contrast to those caused by adding extra foetal calf serum, the latter increasing both background DNA synthesis and viability.

A second property of mouse serum is also brought to light by this experiment. When the index of transformation is measured in mixed cultures it is found to increase as the amount of mouse serum increases.

TABLE 23. Toxic effects of normal mouse serum.

Serum concentrations		Mean DNA synthesis in unmixed cultures		DNA synthesis in mixed cultures	Index of transformation	% CBA cell survival
FCS	Mouse serum	CBA	(CBA x A) _F ₁			
10%	0	1,340 ± 120	890 ± 30	2,390 ± 100	2.2 ± 0.1	100
10%	1%	320 ± 40	300 ± 30	1,160 ± 90	3.7 ± 0.4	96 ± 14
10%	2%	260 ± 40	110 ± 10	620 ± 70	3.3 ± 0.5	93 ± 13
10%	4%	62 ± 5	46 ± 9	340 ± 40	6.3 ± 0.9	81 ± 15
10%	10%	2 ± 5	4 ± 7	4 ± 10	-	27 ± 6
20%	0	1,930 ± 120	1,970 ± 90	4,600 ± 90	2.4 ± 0.1	117 ± 17

CBA and (CBA x A)_F₁ lymph node cells in CM/10% FCS (batch 19) were mixed with either an extra 10% of FCS or with varying amounts of fresh serum obtained by syringe bleeding from 3 anaesthetised ten-month old male CBA mice. The serum was neither heated, nor filtered, but was frozen to -20° for 3 hrs before use. Aliquots of 1.0 million unmixed and mixed cultures were dispensed into the wells of a microtest plate and labelled after 98 hrs with H-3 thymidine for 16 hrs. Figures for DNA synthesis are cp5m/50 microl. of culture.

As shown in Table 24 this ability of low concentration of mouse serum to enhance MLC responses occurs independently of which batch of FCS is used whereas the ability of at least low concentrations of mouse serum to inhibit background DNA synthesis is not apparent with batches of FCS supporting low levels of DNA synthesis. Thus although 2% mouse serum was able to cause

TABLE 24. The effect of mouse serum in the presence of different batches of FCS.

FCS batch	Mouse serum	Mean background in unmixed cultures	Stimulation in mixed cultures	Index
19	0	$4,190 \pm 80$	$2,940 \pm 340$	1.7 ± 0.1
	+2%	$1,960 \pm 50$	$5,010 \pm 370$	3.6 ± 0.2
72	0	430 ± 35	820 ± 150	2.9 ± 0.4
	+2%	420 ± 30	$1,370 \pm 240$	4.3 ± 0.4
73	0	450 ± 45	710 ± 200	2.6 ± 0.5
	+2%	460 ± 30	$1,710 \pm 270$	4.8 ± 0.6
74	0	360 ± 25	560 ± 110	2.6 ± 0.4
	+2%	330 ± 20	$1,240 \pm 200$	4.8 ± 0.6

CBA and (CBA x A)₁F₁ lymph node cells were washed 3 times with saline containing 2% batch 74 FCS, and then resuspended in CMH lacking any serum. Each suspension was divided into two portions to one of which mouse serum was added to give a final concentration of 2%. Both sets of suspension were then subdivided and an appropriate batch of FCS added to each to a final concentration of 20%. Aliquots of 1.0 million cells from mixed and unmixed cultures were dispensed into a microtest plate and incubated for 77 hrs before labelling with H-3 thymidine for 23 hrs. Mouse serum for this experiment was prepared the day before use by syringe bleeding of 3 8-month old anaesthetised CBA mice. It was frozen to -20°C overnight and not heated or filtered. Figures for DNA synthesis are cp5m/50 microl. of culture.

more than 50% inhibition of background DNA synthesis with batch 19 FCS, no detectable inhibition of background DNA synthesis occurred with batches 72, 73 and 74. Yet, mouse serum caused an enhancement of the MLC response with all these batches of FCS. To examine whether the two effects observed were peculiar to CBA serum the experiments shown in Table 25 were performed.

TABLE 25. Specificity tests on the effects of mouse serum.

Serum donors		Concn. of mouse serum	Mean background in unmixed cultures	Stimulation in mixed cultures	Index
Age (months)	Strain				
<u>Experiment 1.</u>					
-	-	0	5,240 $\pm$ 210	14,970 $\pm$ 300	3.8 $\pm$ 0.2
3 $\frac{1}{2}$	CBA	2%	1,100 $\pm$ 50	8,240 $\pm$ 310	8.4 $\pm$ 0.5
11	CBA	2%	1,130 $\pm$ 40	8,540 $\pm$ 540	8.6 $\pm$ 0.6
11	A	2%	1,640 $\pm$ 120	11,030 $\pm$ 320	7.7 $\pm$ 0.6
3 $\frac{1}{2}$	CBA	5%	440 $\pm$ 40	4,690 $\pm$ 180	11.5 $\pm$ 1.0
11	CBA	5%	570 $\pm$ 40	3,770 $\pm$ 180	8.9 $\pm$ 0.9
11	A	5%	800 $\pm$ 30	6,610 $\pm$ 50	9.3 $\pm$ 0.3
<u>Experiment 2.</u>					
-	-	0	5,680 $\pm$ 190	13,590 $\pm$ 700	3.4 $\pm$ 0.2
3 $\frac{1}{2}$ ⁺	CBA ⁺	2%	1,510 $\pm$ 50	9,130 $\pm$ 380	7.0 $\pm$ 0.3
4	CBA	5%	870 $\pm$ 70	4,710 $\pm$ 120	6.4 $\pm$ 0.5
4	C57B1	5%	560 $\pm$ 40	4,730 $\pm$ 290	9.4 $\pm$ 0.8
4	A	5%	1,030 $\pm$ 70	7,280 $\pm$ 290	8.0 $\pm$ 0.6

In both experiments CBA and (CBA $\times$ A) F_1 lymph node cells were cultivated in CM/10%FCS in tubes in the presence of different sources and concentrations of mouse serum. Cultures were labelled after 72 hrs with H-3 thymidine for 16 hrs. In both experiments batch 19 FCS was used (see Table 24). Each kind of serum used in experiment 1 was obtained from syringe bleeding of pools of 2 anaesthetised female mice; the blood was allowed to clot overnight at 4 $^{\circ}$, serum prepared on the morning of the experiment, and frozen to -20 $^{\circ}$ C for 5 hrs before use. In experiment 2, serum was obtained from the blood of 3 CBA, 2 C57B1 and 2 A mice (all 4-month old males) and prepared in exactly the same way as in experiment 1. Figures for DNA synthesis are cp5m/50 microl. of culture.

+ This serum was that used in experiment 1 and kept at 4 $^{\circ}$ for 6 days.

**TABLE 26. Buffering of non-specific effects of mouse serum
by excess FCS.**

Serum concentrations		Mean Background in unmixed cultures	Stimulation in mixed cultures	Index
FCS	Mouse serum			
<u>Experiment 1.</u>				
10%	0	860 $\pm$ 80	1,650 $\pm$ 90	2.9 $\pm$ 0.3
10%	1%	730 $\pm$ 65	2,280 $\pm$ 190	4.1 $\pm$ 0.4
10%	5%	250 $\pm$ 20	990 $\pm$ 310	5.0 $\pm$ 0.3
20%	0	1,320 $\pm$ 110	3,150 $\pm$ 360	3.4 $\pm$ 0.4
20%	1%	1,270 $\pm$ 90	2,750 $\pm$ 50	3.2 $\pm$ 0.5
20%	5%	820 $\pm$ 80	2,440 $\pm$ 210	4.0 $\pm$ 0.5
<u>Experiment 2.</u>				
10%	0	990 $\pm$ 60	1,170 $\pm$ 160	2.2 $\pm$ 0.2
10%	5%	290 $\pm$ 25	600 $\pm$ 50	3.1 $\pm$ 0.3
10%	10%	195 $\pm$ 20	240 $\pm$ 50	2.3 $\pm$ 0.3
20%	0	730 $\pm$ 30	1,410 $\pm$ 70	2.9 $\pm$ 0.2
20%	5%	390 $\pm$ 30	730 $\pm$ 50	2.9 $\pm$ 0.3
20%	10%	295 $\pm$ 20	450 $\pm$ 30	2.5 $\pm$ 0.2

In both experiments CBA and (CBA $\times$ A)_F₁ lymph node cells were cultured in unmixed and mixed culture in CMH containing the serum concentrations shown. Cultures were labelled after 72 hrs culture for 16 hrs. Batch 72 FCS was used in both experiments. Mouse serum was obtained fresh from the blood of 2 15-month old CBA males (experiment 1) and 4 $3\frac{1}{2}$ -month old CBA males (experiment 2). In all cases mice were anaesthetised with nembutal and bled from the heart with a syringe.

It therefore seems that mouse serum contains two kinds of factors one of which is selectively toxic to those cells responsible for the greater part of background transformation, the other kind promoting the transformation of those cells that respond in culture to MLC antigens. At high (greater than 5%) concentrations of mouse serum the toxic factors cease to be selective and all mouse lymphocytes are killed. Such a toxicity to autologous cells could not exist in vivo and it must be concluded that at least the toxic factors are products of autolysis, oxidation, or some other chemical change that occurs at or after bleeding.

These non-specific effects would present a considerable problem when testing mouse serum for the possible presence of blocking factors. Therefore experiments were done to see whether using higher concentrations of FCS would "buffer" the non-specific effects (Table 26). Both experiments showed that in the presence of 20% FCS both the toxic and MLC enhancing effects of normal mouse serum were reduced compared to those observed in the presence of 10% FCS. As a supplement to each of these experiments a comparison was made between two different methods of bleeding. One method was that used in all other experiments described in this section - namely, mice were anaesthetised with nembutal and bled from the heart by cardiac puncture with a syringe. The second method was that used by Festenstein (see Materials and Methods). The effects of serum obtained from blood prepared by these two methods is shown in Table 27. Although there was no difference between the toxic properties, serum obtained by the method of Festenstein had distinctly less MLC enhancing properties, and although an amount of enhancement still occurred the major part of this effect was abolished. As a result of the experiments described in Tables 26 and 27, virtually all MLC tests on serum from tolerant or unresponsive mice were performed with serum obtained by the Festenstein method and in the presence of 20% FCS.

As a result enhancing effects were only observed occasionally.

However, the toxic effects of mouse serum were still so high that the maximum concentration of mouse serum that could be used in cultures was about 6%. Cytotoxic assays being conducted over a much shorter culture period than MLC were less susceptible to non-specific effects of mouse serum, as shown in Table 28. In a 7 hr assay cytotoxicity by in vitro sensitised cells was reduced by only about 50% even with 11% normal mouse serum, and in other experiments it has been possible to use 20% mouse serum successfully.

TABLE 27. Serum prepared from blood obtained by two different methods.

FCS batch	Mouse serum	Mean background in unmixed cultures	Stimulation in mixed cultures	Index
<u>Experiment 1.</u>				
72	0	990 $\pm$ 60	1,170 $\pm$ 160	2.18 $\pm$ 0.20
72	5% ^(a)	290 $\pm$ 25	600 $\pm$ 50	3.07 $\pm$ 0.30
72	5% ^(b)	290 $\pm$ 30	370 $\pm$ 50	2.27 $\pm$ 0.27
<u>Experiment 2.</u>				
19	0	17,430 $\pm$ 990	12,080 $\pm$ 1,530	1.74 $\pm$ 0.12
19	2% ^(a)	3,560 $\pm$ 160	6,070 $\pm$ 620	2.70 $\pm$ 0.21
19	2% ^(b)	3,850 $\pm$ 80	4,870 $\pm$ 420	2.26 $\pm$ 0.12

(a) = Mice bled while under nembutal anaesthetisation.

(b) = Mice bled by cutting open the heart after death by nitrogen asphyxiation.

In both experiments CBA and (CBA $\times$ A)_F₁ lymph node cells were cultured in mixed and unmixed cultures for 72 hrs, then labelled with H-3 thymidine for 16 hrs. Two different batches of FCS were used at a concentration of 10% and mouse serum, obtained by two methods of bleeding, added to certain cultures. Serum was in both cases prepared fresh from 3½-month old (experiment 1) or 15-month old (experiment 2) CBA males, and was neither heated nor filtered.

TABLE 28. Effect of normal mouse serum on cytotoxic killing.

Mouse serum	Spontaneous release	Specific cytotoxic release	% Cytotoxicity
0	1,040 $\pm$ 40	1,850 $\pm$ 90	23.7 $\pm$ 1.2
2%	ND	1,460 $\pm$ 100	18.7 $\pm$ 1.3
11%	1,010 $\pm$ 30	870 $\pm$ 70	11.1 $\pm$ 0.9

CBA lymph node cells were cultured with irradiated CBA or irradiated C57Bl spleen cells for $4\frac{1}{2}$ days in MLC at a 2:1 responder:stimulator ratio. After harvesting they were recultured with labelled EL4 cells for 7 hrs at an effector:target cell ratio of 50:1 in CMH/20% FCS. Serum was obtained from two 6-month old CBA males bled after asphyxiation and stored at -20° for 5 days. Figures for cytotoxic release are cp5m/100 microl of supernatant.

The inhibitory effects of normal mouse serum on in vitro responses have been observed previously (426-428). It has been postulated that the inhibitory effects may be due to the presence of regulatory proteins in normal mouse serum (427,428) similar perhaps to the regulatory γ -globulins described by Cooperband et al. (429). However, in the present study such explanations are not acceptable since mouse serum was clearly toxic to cultured lymphocytes (Table 22). Since such toxic properties cannot be acting in vivo it seems likely that toxic materials are produced as a result of denaturation of the serum during preparation, although the possibility that cultured lymphocytes become susceptible to a normal component of serum cannot be ruled out. The ability of mouse serum to enhance MLC responses at low concentrations can, however, be exploited. Indeed, two recent publications of MLC methods have advocated the inclusion of small amounts of mouse serum as medium supplement (351,430).

TABLE 29. The ability of male and female CBA lymph node cells to respond in MLC.

Responder cell	Background (syngeneic culture)	Stimulation by $[(CBA \times A)F_1]$ cells x	Stimulation by $[(CBA \times DBA/2)F_1]$ cells x	Index to $[(CBA \times A)F_1]$ cells x	Index to $[(CBA \times DBA/2)F_1]$ cells x
Male	2,600 $\pm$ 170	3,220 $\pm$ 350	16,360 $\pm$ 230	2.24 $\pm$ 0.19	7.29 $\pm$ 0.48
Female	5,490 $\pm$ 200	4,270 $\pm$ 330	17,600 $\pm$ 660	1.78 $\pm$ 0.08	4.21 $\pm$ 0.19

CBA lymph node cells prepared from pools of 4 male and 4 female mice of identical age were mixed in a 2:1 ratio with irradiated CBA, $(CBA \times A)F_1$ lymph node cells from male mice. Aliquots containing 1.0 million responder cells were dispensed into microtest plates and labelled after 73 hrs with H-3 thymidine for 17 hrs. Figures for DNA synthesis are cp10m/50 microl. of culture.

3. Effect of mouse age and sex on the ability of lymphocytes to respond and stimulate in MLC.

(a) Sex.

The comparative ability of male and female cells to respond in MLC was investigated in an experiment shown in Table 29. The stimulations obtained against two strains of antigen were similar in both male and female responder cells. However, because of the higher background in female cells the indexes were significantly higher for male responder cells than for female. The higher background in female cells may be related to the syngeneic effects found in one experiment (part of the same experiment shown here) in which irradiated female lymph node cells promoted higher levels of background DNA synthesis in syngeneic cells, either male or female, than did male cells (Chapter 3, Section 9b). Because of this difference in background transformation the experiment shown here is inconclusive. However, in an extensive study of responses between male and female cells Bach and colleagues (367) could find no evidence for different levels of responsiveness in male and female cells and therefore in this study responder cells were obtained from either male or female mice. In experiments where the responsiveness of cells from tolerant or unresponsive mice was tested control cells were always obtained from sex-matched mice.

(b) Age.

Table 30 shows the results of 3 experiments where the effects of the age of the lymph node cell donor on the reactivity of the cells in MLC was studied. The experiment where cells from different aged mice were examined for stimulatory power failed to show any difference between 'old' and 'young' cells. This suggests that there is no decrease of immunogenicity of MLC antigens with aging, at least in

TABLE 30. The effect of donor age on the performance of lymphocytes in MLC.

A. Effect of donor age on response in MLC.

Age of donors of responder cells (months)	Stimulation	Index			% response relative to that at 2 months	
		Response to (CBA x A)F ₁ cells				
	Experiment No. 1	3	1	3	1	3
2	4,500 ± 120	3,350 ± 340	3.50 ± 0.17	1.95 ± 0.16		
6	1,920 ± 160	640 ± 150	2.20 ± 0.13	1.55 ± 0.17	48 ± 6	58 ± 20
12-13	1,640 ± 160	1,480 ± 220	1.71 ± 0.08	1.26 ± 0.04	28 ± 4	27 ± 4
		Response to (CBA x DBA/2)F ₁ cells				
	Experiment No. 2	3	2	3	2	3
2	4,640 ± 160	14,390 ± 590	6.5 ± 0.4	5.1 ± 0.4		
6	3,880 ± 220	8,400 ± 130	6.7 ± 0.5	8.2 ± 0.8	103 ± 12	175 ± 26
12-13	1,440 ± 200	9,280 ± 680	5.6 ± 0.7	3.1 ± 0.2	84 ± 14	51 ± 7

B. Effect of donor age on stimulation in MLC.

Age of donors of responder cells (months)	Age of donors of stimulating cells (months)	Stimulation in mixed cultures	Index
2	2	14,390 ± 590	5.1 ± 0.4
	13	20,350 ± 500	5.9 ± 0.3
13	2	9,280 ± 680	2.6 ± 0.1
	13	12,210 ± 320	3.1 ± 0.2

For experiments in Section A, lymph node cells from healthy CBA female mice of various ages were cultured in MLC with lymph node cells from 2-month old (CBA x A)₁F₁ or (CBA x DBA/2)₁F₁ mice. Experiments 1 and 2 were performed with live stimulator cells, experiment 3, with irradiated stimulator cells. In the second part of experiment 3 (Section B), CBA cells were stimulated with (CBA x DBA/2)₁F₁ cells from either 2 or 13-month old mice. All cultures were labelled after 72-78 hrs with H-3 thymidine for 16 hrs. Figures for DNA synthesis are cp10m/50 microl. of culture. Relative responses were calculated as:-

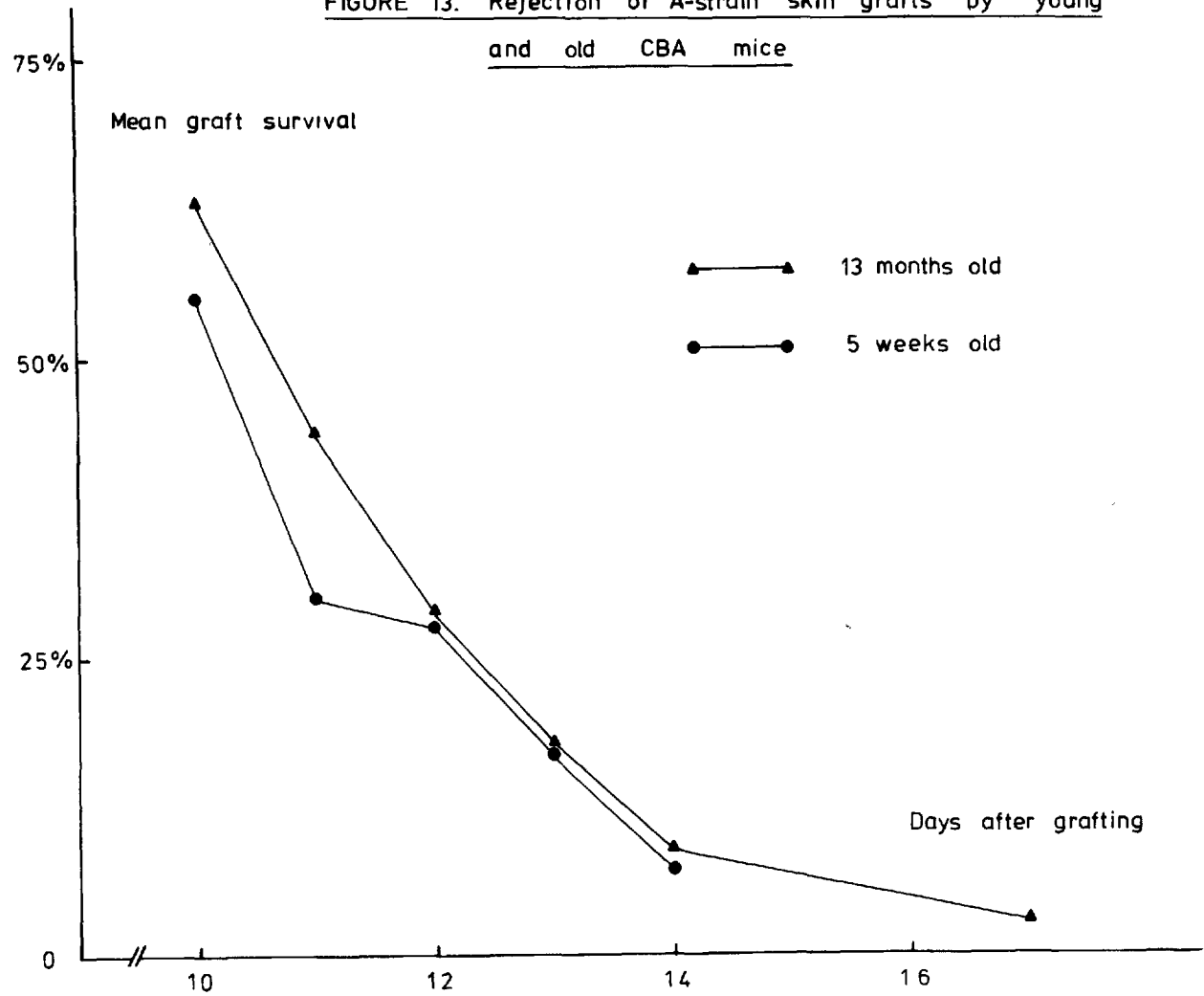
$$F = \frac{I_x - 1}{I_{2m} - 1} \times 100 \%$$

where I_x is the index for 6-month or 12-13 month-old responder mice and I_{2m} is the index for 2-month old responder mice.

the strain used. By contrast the ability of CBA lymph node cells to respond to (CBA x A) F_1 cells showed a large decline with increasing age, cells from 12-month old mice having only about $\frac{1}{4}$ of the reactivity of cells from 2-month old mice. However, the pattern of declining MLC responsiveness was not the same for all antigens: thus, in contrast to the situation with A-strain antigens, there was no evidence that 6-month old CBA mice had any weakening of their responsiveness to DBA/2-strain antigens and, although a decline in responsiveness had occurred by 12-13 months of age, it was not to such low levels as occurred in the response to A-strain alloantigens. Possibly the participation of the H2 LD-1 locus in the CBA anti-DBA/2 response but not in the CBA anti-A response, where there is disparity only at the minor H2 locus, explains the different aging patterns - clones directed against the major LD locus antigens may be lost more slowly during aging than clones that recognise weak LD antigens.

To examine whether the large decline in MLC responsiveness to A strain cells leads to a weakening of the ability of old CBA mice to reject A strain allografts 15 (8 female, 7 male), 5 week old CBA mice and 17 (9 male, 8 female), 13-month old CBA mice were grafted with A strain tail skin. The progress of graft rejection in the two groups is shown in Figure 13. There is a suggestion that the early rate of graft rejection in the old mice was somewhat less than in the young mice, possibly due to a weaker ability of cells from old mice to enter into the inductive phase of the immune response. However, the differences between the two curves are very small compared to the differences in MLC reactivity between the cells from such mice. This may be because the rejection of H2 incompatible grafts is a rather insensitive measure of immune reactivity, or because levels of MLC reactivity in vitro cannot be directly correlated with allograft responses in vivo.

FIGURE 13. Rejection of A-strain skin grafts by young
and old CBA mice

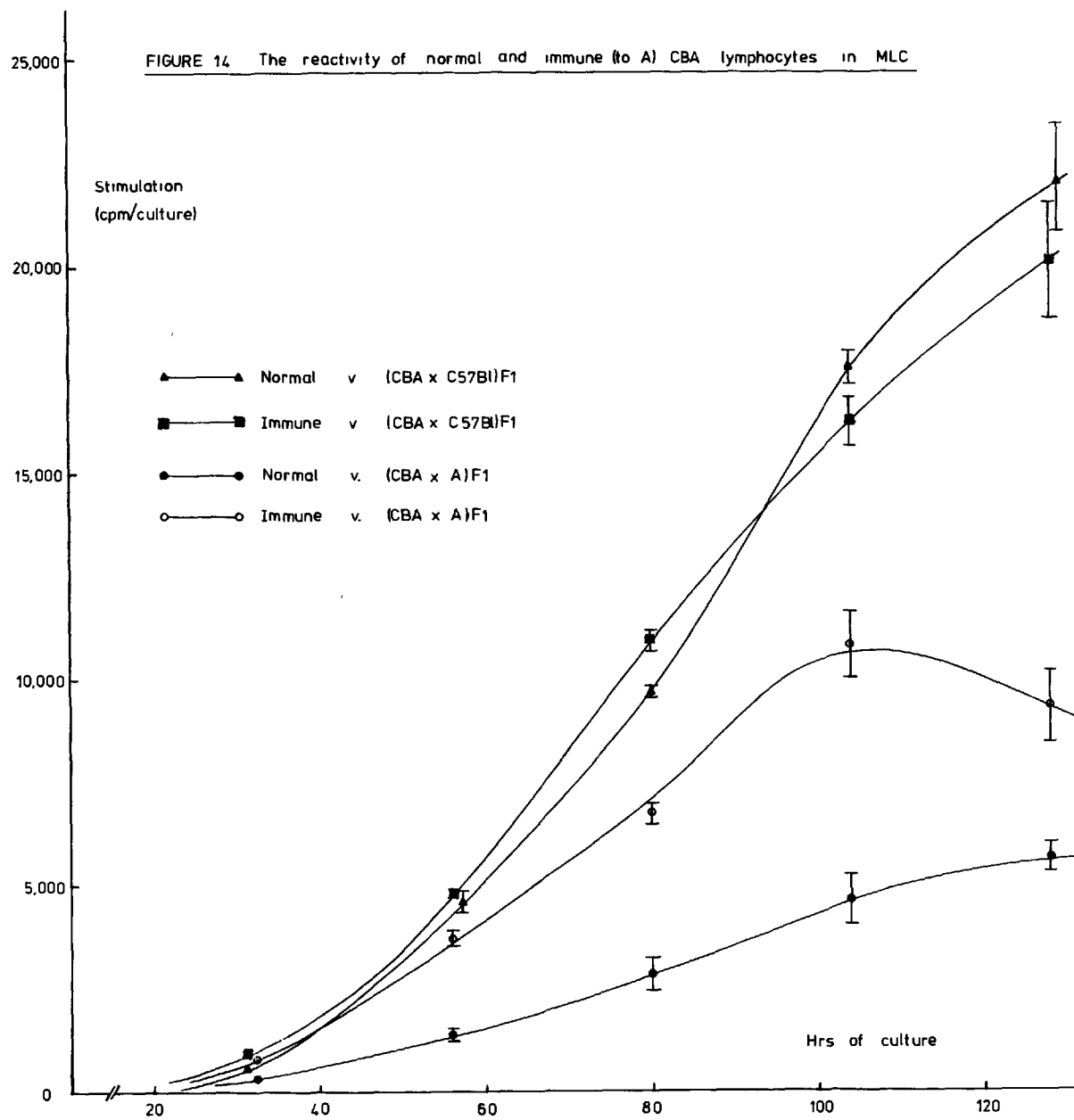


A decline of immune responses with increasing age has been found by many workers and the possibility that aging is in fact a consequence of a decreased efficiency of the immune system has recently been reviewed by Walford (431). The importance of the phenomenon as regards MLC tests for cellular reactivity is that a true comparison between cells from different animals can only be valid if the donors are of the same age. Age matching in the CBA v. A combination is particularly important and in all experiments in this thesis great care was taken to ensure that animals donating either responder or stimulator cells were of the same age.

4. Detection of pre-sensitisation by MLC.

(a) Cellular response.

As recently discussed by Wilson et al (409), the pool of cells which initiate GVH and MLC responses against major histocompatibility antigens is not subject to the same degree of expansion following immunisation as are cells reactive to other types of antigen. Thus, although pre-immunisation of rats (432), mice (433), and humans (434) leads to both increased magnitude and speed of the subsequent MLC reaction in vitro, the degree of augmentation is small and also short lived, being detectable for only a few weeks following immunisation. Furthermore, both the route of administration of antigen and the source of the test cells is important. For example, whereas subcutaneous immunisation increases the MLC reactivity of rat peripheral blood cells, no increased reactivity is found in the thoracic duct (409). Indeed, intravenous immunisation of rats (432,435) or skin grafting of dogs (436) causes decreased MLC reactivity in the peripheral blood. A rationalisation of these perplexing observations has been provided by Larner (435) who found that the decrease of MLC-reactive cells in the blood was mirrored by an increased reactivity in the spleen and



he proposed that the primary effect of immunisation is to cause a redistribution of MLC-reactive cells. Undoubtedly, other factors are also important, Bondevik and Thorsby (43₄) having made the interesting observation that the augmented MLC reactivity in peripheral blood following intradermal immunisation of human volunteers was apparently due to increased responsiveness against SD rather than against LD antigens. Clearly, further investigation of the effects of immunisation on MLC reactivity is required, preferably in congenic recombinant mice. In order to examine whether a state of pre-sensitisation could be detected in skin-grafted and ip immunised mice using the culture system described in Chapter 3 the following experiments were performed.

In the first experiment, 8 6-week old CBA female mice were chosen: 4 were grafted with A-strain skin and 4 remained as ungrafted controls. 18 days later peripheral and mesenteric nodes were removed from 2 grafted and 2 control mice. These were cultured in MLC with (CBA x A) F_1 and (CBA x C57B1) F_1 lymph node cells. Cultures were labelled with H-3 thymidine for 16-18 hr periods beginning 25, 48, 72, 97, and 120 hrs after the start of incubation. The stimulation observed in mixed cultures is shown in Figure 14. Mice grafted with allogeneic skin have an elevated response to cells bearing alloantigens of the graft donors but not to cells of a third party strain. On each day of assay except the last the MLC response of immune cells was more than twice that of normal cells. Thus, a specific sensitisation is detectable in this MLC system. Furthermore, it appears that the peak response in the immune cells occurs earlier than the peak response in normal cells, although the early peak is not so clearly defined as in some other systems (434-436).

2 months later the MLC test was repeated on the remaining grafted and ungrafted mice with the same methodology as described above except that cultures were labelled after 89, 113, and 137 hrs for 8 hrs. At none of these times was there any detectable difference between the normal and immune cells. In Table 31 the results for the two experiments are shown for day 4-5 when the difference between normal and immune cells at 18 days post grafting was maximal. An interesting point is that the background transformation of immune cells at 18 days is significantly higher than that of normal cells. If this were due merely to slightly different initial cell concentrations an elevated response of immune cells to all antigens would be expected, yet this was not observed. The implication is that 18 days after grafting some proliferation is still occurring in the lymph nodes.

TABLE 31. The behaviour of cells from immunised mice in MLC. I.

Days after grafting	Donor of CBA cells ⁺	Background transformation in CBA lymph node cells	Stimulation by (CBaxA)F ₁ cells	Stimulation by (CBaxC57)F ₁ cells	Factor of immunisation
18	N	8,300 $\pm$ 550	11,600 $\pm$ 1,500	43,900 $\pm$ 1,000	2.3 $\pm$ 0.3***
	I	12,500 $\pm$ 230	27,000 $\pm$ 1,900	40,600 $\pm$ 1,400	
80	N	10,700 $\pm$ 650	17,600 $\pm$ 1,200	35,100 $\pm$ 940	1.0 $\pm$ 0.1 ^{NS}
	I	8,600 $\pm$ 500	16,900 $\pm$ 1,000	41,700 $\pm$ 1,200	

The method used for testing the cells in MLC is described in the text. Cells from 18-day immune mice were labelled after 97 hrs culture for 16 hrs; cells from 80-day immune mice after 89 hrs for 8 hrs. Figures for DNA synthesis are cp5m/100 microl. of culture. Factor of immunisation = response of immune cells to specific antigen/response of normal cells. The significance of the difference from 1.0 is shown.

- + I = lymph node cells from pools of 2 mice grafted with A-strain skin.
N = lymph node cells from pools of 2 normal mice.

TABLE 32. Behaviour of cells from immunised mice II.

Days after grafting	Cells ⁺ tested	Status of donor	Background transformation of CBA cells	Stimulation by (CBA x A) _F ₁ cells	Factor of immunisation ⁺⁺
17	MLN	N	15,780 ± 600	5,530 ± 520	0.88 ± 0.17 ^{NS} 1.50 ± 0.16 ^{**}
		I-ip	18,280 ± 700	4,850 ± 810	
		I-G	15,720 ± 550	8,280 ± 410	
	DLN	N	18,450 ± 580	10,950 ± 340	1.46 ± 0.12 ^{***}
		I-G	25,900 ± 800	16,000 ± 1,160	
37	MLN	N	4,480 ± 210	4,990 ± 210	1.99 ± 0.10 ^{***} 1.43 ± 0.08 ^{***}
		I-ip	3,900 ± 200	9,930 ± 260	
		I-G	4,940 ± 60	7,180 ± 230	
79	MLN	N	5,540 ± 170	5,900 ± 290	0.91 ± 0.06 ^{NS} 1.05 ± 0.07 ^{NS}
		I-ip	5,360 ± 110	5,340 ± 190	
		I-G	5,560 ± 240	6,180 ± 280	

Thirty 2-month old CBA female mice were chosen from the same stock box and kept together in the same box throughout the whole of this experiment. 12 mice were grafted with A-strain tail skin, 12 were injected intraperitoneally (ip) with 10 million (total) (CBA x A)_F₁ spleen cells, and 6 mice were not treated. Pairs of mice were sacrificed at times shown in the Table. In the test at 17 days mesenteric and draining peripheral nodes were removed from pairs of grafted and normal mice and separate cell suspensions made from the two types of nodes. These and mesenteric lymph node cells from 2 mice immunised ip, were cultured alone and in a 1:1 ratio with (CBA x A)_F₁ spleen cells. Cultures were labelled after 97 hrs for 20 hrs. In the tests at 37 and 79 days, mesenteric lymph nodes only were removed from pairs of mice and cultured with (CBA x A)_F₁ spleen cells at a 3:1 responder:stimulator ratio. Cultures were labelled after 72 hrs for 18 hrs. All figures for DNA synthesis are cp10m/50 microl. of culture.

+ MLN = mesenteric lymph node, DLN = draining peripheral lymph nodes (or corresponding nodes in normal mice).

++ Factor of immunisation and statistical tests are calculated as in Table 31.

The results of a detailed repeat experiment are shown in Table 32, 33 and 34. The results of Table 32 confirm those of Table 31. 17 days after immunisation lymph node cells from grafted mice showed elevated MLC responsiveness; this was also easily detectable at 37 days, but by 79 days the cells behaved like normal cells. The results with draining lymph node cells from mice grafted 17 days earlier are particularly interesting. Such cells showed a

distinct elevation of background transformation compared with normal peripheral lymph node cells. As mentioned above, in the first experiment (Table 31), cells prepared from peripheral and mesenteric lymph nodes of grafted mice showed elevated background transformation in 18 days post-grafting. It thus appears that this elevated background is due to increased proliferation of the cells from the draining lymph nodes of grafted mice. The nodes are indeed grossly enlarged at this time. The practical effect of this is to lead to an underestimation of the MLC response if the index of transformation is used as the guiding parameter. Indeed, if the cells causing the increased background are specific antigen-reactive cells, such that the proliferation represents the continuation of an in vivo MLC reaction, the MLC stimulation in culture is also underestimated. That the factor of immunisation for the draining lymph node cells was the same as that for mesenteric lymph node cells may support the idea that the stimulation in the former cells is being underestimated.

In contrast to mice immunised by grafting, the mesenteric nodes from mice immunised by ip injection of 10 million (CBA x A) F_1 cells did not show evidence of sensitisation at 17 days. However, strong sensitisation was apparent at 37 days, and, as with grafted mice, this had disappeared by 79 days. In conclusion, although the effects of presensitisation are apparent in MLC, they can be detected for only a relatively short time.

As a second part to the experiment described in Table 32, cells from immunised and normal mice were cultured in MLC for $4\frac{1}{2}$ days, and then tested for cytotoxic activity against Cr-labelled A lymphoma cells. The results are shown in Table 33.

TABLE 33. Behaviour of cells from immunised mice III.

Days after grafting	Status of donor	Cells from	Sensitised in MLC against:	% killing of A strain lymphoma in CML test	Significance of difference between N & I
17	N	MLN + DLN	-	0(control)	
	I-G	MLN + DLN	-	8 + 1***	
	N	MLN + DLN	(CBA x A) ₁ F ₁	39 + 3	
	I-ip	MLN	(CBA x A) ₁ F ₁	72 + 2	***
	I-G	MLN + DLN	(CBA x A) ₁ F ₁	68 + 2	***
37	N	MLN	-	0(control)	
	I-G	MLN	-	1 + 2 ^{NS}	
	N	MLN	(CBA x A) ₁ F ₁	62 + 2	
	I-ip	MLN	(CBA x A) ₁ F ₁	82 + 2	***
	I-G	MLN	(CBA x A) ₁ F ₁	70 + 3	*

The lymph node cell suspensions used in the MLC tests in Table 32 were cultured either alone or with (CBA x A)₁F₁ spleen cells at a responder: stimulator ratio of 3:1. 4½ days later cultured cells were harvested, washed, and recultured with Cr-labelled A lymphoma cells for 7 hrs at an effector: target cell ratio of 150:1. In the test done at 17 days mesenteric and draining lymph node cells from grafted and normal mice were pooled.

At both 17 and 37 days after immunisation cells from immune mice showed significantly higher cytotoxicity than cells from normal mice after sensitisation in MLC. Cells from 17 day grafted mice showed significant killing of target cells after culture in the absence of further antigen, but by 37 days this activity had become undetectable.

The results of Table 33 show that a state of presensitisation in lymphoid cells can lead to increased generation of cytotoxic cells when they re-encounter antigen in MLC. An interesting finding was that lymph node cells from mice immunised ip 17 days previously did not show elevated MLC responsiveness yet showed increased cytotoxic activity after MLC. This dissociation between the two phenomena is no doubt a reflection of the fact that cytotoxic cells detected in the CML assay are directed against antigens distinct from those that induce the bulk of DNA synthesis in MLC (151, 367, 368).

TABLE 34. The effects of sera from immune mice on the MLC responsiveness of normal cells.

Days after immunisation	Status of donor	Background in control culture	Stimulation in mixed culture	Index	% Relative ⁺⁺ to normal	Haemagglutination titre, 2 ⁿ
<u>Experiment A.</u>						
-	N	3,030 ± 180	8,140 ± 560	3.70 ± 0.27		0
6	I-ip	5,000 ± 230	9,480 ± 780	2.90 ± 0.20	70 ± 10	10
	I-G	3,640 ± 170	7,720 ± 670	3.16 ± 0.23	80 ± 12	8
10	I-ip	4,260 ± 110	5,890 ± 560	2.39 ± 0.14	51 ± 7	10
	I-G	4,240 ± 230	6,480 ± 450	2.54 ± 0.16	57 ± 8	8
17	I-ip	4,710 ± 270	5,230 ± 420	2.12 ± 0.14	41 ± 7	8
	I-G	3,990 ± 290	3,670 ± 540	1.92 ± 0.18	34 ± 7	10
	H ⁺	1,820 ± 180	890 ± 200	1.49 ± 0.15	18 ± 6	ND
<u>Experiment B.</u>						
-	N(37d)	3,140 ± 160	4,420 ± 250	2.41 ± 0.14		0
-	N(79d)	2,820 ± 90	4,000 ± 220	2.42 ± 0.11		
17	I-G	2,600 ± 150	2,580 ± 310	1.99 ± 0.15	70 ± 12	10
37	I-ip	2,780 ± 140	3,490 ± 270	2.26 ± 0.14	89 ± 11	7
	I-G	2,770 ± 120	3,570 ± 170	2.29 ± 0.11	91 ± 10	4
79	I-ip	3,250 ± 130	3,720 ± 270	2.14 ± 0.11	81 ± 9	5
	I-G	3,110 ± 130	2,750 ± 280	1.88 ± 0.11	62 ± 9	7
129	I-ip	ND	ND	ND	ND	<1
	I-G	ND	ND	ND	ND	>7
	H ⁺	1,940 ± 90	1,540 ± 160	1.79 ± 0.11	56 ± 9	13½

For experiment A sera were obtained by bleeding pairs of mice 6, 10, and 17 days after immunisation, one pair of normal mice being bled at 17 days. Blood was allowed to clot overnight at 4°, serum separated next morning, spun twice, ampouled and frozen to -20°. All sera were unfrozen 13 days after the last bleeding and tested for their effects on the MLC response of CBA lymph node cells cultured with irradiated CBA and A spleen cells at a responder:stimulation rate of 2:1. Mouse serum concentration was 3.7% and cultures were labelled after 72 hrs for 23 hrs. For experiment B sera were obtained from pairs of normal and immune mice 37 and 79 days after immunisation. Blood was allowed to clot for 6 hrs at 4°, serum prepared, left overnight at 4°, then ampouled and frozen to -80°. Sera were unfrozen 80 days after the last bleeding and tested on cultures as in experiment A, except that the labelling period was only 17 hrs. Serum obtained 17 days after grafting which had been stored at -80° since experiment A was retested to provide a positive control in the second experiment. For other details see Table 32.

+ H = hyperimmune CBA anti A sera which had been stored for some months at -80°.

++ The relative response for serum x was calculated as $(I_x - 1/I_N - 1) \times 100\%$, where I_x and I_N were the indexes with serum x and normal serum respectively.

In a third part of the experiment described in Tables 32 and 33, blood was taken from mice at various times after immunisation and tested for ability to inhibit the MLC response between normal cells. The sera were frozen and tested in two separate MLC experiments, as shown in Table 34. Inhibition was apparent with all immune sera. The degree of inhibition was similar for serum from mice immunised by a skin graft and mice immunised by injection of spleen cells, and in both cases, reached a peak at around 17 days. Unfortunately, no direct comparison is possible between experiments A and B because the positive controls in the latter experiment gave much lower inhibition than in the former. Nevertheless, it is clear that at 37 days there was less inhibitory activity than at 17 days. Strangely, at 79 days there seems to have been a regeneration of inhibitory activity, at least in the grafted mice. That this was not a technical error was confirmed by the haemagglutination tests which showed that a second cycle of antibody synthesis began between 37 and 79 days after grafting and continued until at least 129 days post grafting. A second cycle of antibody synthesis was not observed in mice immunised with spleen cells.

By contrast, early in the antibody response there was not such a correlation between MLC inhibiting activity and haemagglutinating antibody. Experiment A shows that with increasing time after immunisation, mouse serum caused increasing inhibition of MLC, yet over the same period there was little change in the level of haemagglutinating antibody. A similar lack of correlation between MLC inhibiting antibody and cytotoxic antibody has been reported (437-439). Further evidence against the involvement of cytotoxic antibody in the phenomenon is provided by the fact that complement is not required and that $F(ab')_2$ antibody fragments are as effective as whole antibody (440).

Many workers have reported that IgG is the main MLC-inhibitory component present in hyperimmune and multiparous sera. Indeed, Milton et al (441) found that the inhibitory activity was predominantly a property of the IgG_{2a} fraction of rat hyperimmune sera. Since only small amounts of IgM antibody are present in such sera, however, the possibility that IgM can also inhibit in MLC if present at high concentration has not been eliminated. The finding here, that at 6 days post immunisation there were high quantities of haemagglutinating antibodies (almost certainly IgM) yet only minimal inhibition, however, supports the notion that IgM is relatively ineffective at inhibiting MLC responses.

In the experiments in Table 34, hyperimmune sera prepared several months previously and stored at -80° (experiment A) or -20° (experiment B) were used as positive controls. They showed increased non-specific toxicity to mouse cells in culture compared to the test sera which had been stored for shorter periods in frozen form. This effect of storage presents problems, and in order to study the effect of hyperimmune serum on MLC responses it was essential to have a control serum prepared and stored under identical conditions. CBA anti-C57B1 hyperimmune serum was raised in 3-4 month old CBA female mice by intraperitoneal injection of 25 million (CBA x C57B1) F_1 spleen cells at weeks 0 and 4, followed by a further injection of 5 million cells at week 6 and bleeding by the method of Festenstein (see "Materials and Methods" and this Chapter, Section 2) 12 days later. F_1 cells as opposed to parental cells were used as immunogen to avoid any possibility of anti-host antibodies being produced by GVH reaction. An identical group of CBA mice were injected at the same times as the first group with the appropriate number of syngeneic CBA spleen cells and bled at the same time. Blood was allowed to clot overnight at 4° and frozen to -80° in sealed ampoules. 6 weeks later aliquots of hyperimmune and control sera were unfrozen and tested for their effect on MLC.

TABLE 35. The effects of hyperimmune serum on mouse MLC responses.**a. Syngeneic control.**

Serum present	CBA v. CBA _x	C57 v. C57 _x
anti CBA	2,100 $\pm$ 60	1,420 $\pm$ 50
anti C57B1	2,080 $\pm$ 80	1,290 $\pm$ 70
	NS	NS

b. Effect on response of CBA cells to various strains of stimulator cell.

Stimulating antigen	Serum present	Observed stimulation	% of control ⁺	significance of difference ⁺⁺
C57B1 _x	Anti-CBA	3,630 $\pm$ 220	27 $\pm$ 3	***
	Anti-C57B1	990 $\pm$ 85		
A _x	Anti-CBA	2,100 $\pm$ 140	80 $\pm$ 9	*
	Anti-C57B1	1,690 $\pm$ 140		
Balb/c _x	Anti-CBA	3,170 $\pm$ 150	66 $\pm$ 6	***
	Anti-C57B1	2,100 $\pm$ 160		

c. Effect on response of C57B1 cells to various strains of stimulator cells.

Stimulating antigen	Serum present	Observed stimulation	% of control ⁺	significance of difference ⁺⁺
CBA _x	Anti-CBA	3,220 $\pm$ 210	101 $\pm$ 8	NS
	Anti-C57B1	3,240 $\pm$ 170		
A _x	Anti-CBA	3,800 $\pm$ 180	95 $\pm$ 6	NS
	Anti-C57B1	3,600 $\pm$ 120		
Balb/c _x	Anti-CBA	2,810 $\pm$ 70	93 $\pm$ 6	NS
	Anti-C57B1	2,620 $\pm$ 140		

CBA lymph node cells were cultured with irradiated CBA (syngeneic control), C57B1, A, and Balb/c strain lymph node cells at 2:1 responder:stimulator ratio in the presence of either CBA anti-CBA, or CBA anti-C57B1 hyperimmune serum, at a concentration of 3.2% (for preparation, see text). A similar series of cultures were done with C57B1 lymph node responder cells. Cultures were labelled after 73 hrs for 20 hrs. All figures for DNA synthesis are cp5m/50 microl. of culture.

+ Calculated as (stimulation with anti-C57B1 serum/stimulation with anti-CBA serum) x 100%.

++ 1-tail t-test.

As shown in Section A of Table 35, there was no significant difference between the background transformations of either CBA or C57B1 cells in the presence of control and hyperimmune sera. Thus, there are no nonspecific effects to be taken into account, and as a result the stimulation obtained in mixed culture can be used directly as a measure of response rather than the index of transformation.

As expected, the anti-C57B1 hyperimmune serum caused a strong inhibition of the CBA anti-C57B1 MLC response. In parallel with the findings of others (438,439) there is partial non-specificity, the responses to A and Balb/c strain cells being inhibited significantly, but less so than the response against the specific antigen (in both cases $P < 0.001$). Interestingly, the inhibition against third party cells does not correlate with known HL-A antigens in man (442) or AgB antigens in the rat (443) indicating that the target antigens are distinct from those that induce cytotoxic or haemagglutinating antibodies.

Most workers have also found that hyperimmune and multiparous antisera can inhibit the ability of cells of the immunising strain to respond in MLC, two recent reports demonstrating this phenomenon in the mouse (444,445). The most obvious explanation of this effect is that the antibodies sterically inhibit the binding of antigen to receptor sites. However, this cannot be the whole truth since as shown in Table 35 and in the experiments of others (441) antisera which can strongly block the ability of cells to stimulate do not necessarily inhibit the same cells from responding. Species variations and strain variations cannot be responsible for the discrepancies since Tyan (444) found that CBA anti-C57 serum in his hands did block the ability of C57 cells to respond in MLC. Presumably differences in the specificities and quantities of antibodies present in different sera, or variations in culture conditions are important.

CHAPTER 5. Studies of neonatally-induced tolerance to trans-plantation antigens in mice.

Introduction.

As early as 1929 Danford and Foster (446) found that skin allografts transplanted to newborn chickens were retained for unusually long periods. However, the recognition that this phenomenon was due to an interference with normal immunological processes awaited the discovery that not only were dizygotic twin cattle born with a stable red cell chimaerism (447) but that such calves were usually unable to reject skin grafts from their twin siblings (448). These and other experiments led Burnet and Fenner (449) to predict that by injecting foreign antigen into embryonic animals it should be possible to reproduce the chimaerism phenomenon in the laboratory. This prediction was amply verified by the experiments of Billingham, Brent and Medawar (4), who showed that injection of 15-day embryonic mice with cells from adult allogeneic mice led in many cases to a prolonged unresponsiveness (tolerance) to donor strain, but not third party, grafts. Most importantly, they found that when cells from pre-sensitised syngeneic mice were injected into tolerant recipients there was rapid breakdown of the allogeneic skin grafts. This demonstrated, in agreement with Owen's detection of donor antigens on the red cells of cattle chimaeras (447), that the retention of allogeneic tissues was not due to their having lost their antigenicity. Further studies showed that normal cells too could induce rejection of tolerated grafts, and Billingham et al proposed that tolerance to alloantigens was due to a central failure of the immune response (450).

Tolerance induction with allogeneic cells is critically dependent on the immunological maturity of the recipients. Thus, Billingham et al (450) reported that embryonic mice greater than

18 days old were less easily made tolerant than embryonic mice younger than 18 days. Subsequent studies showed that although newborn mice could be made tolerant by intravenous injection of allogeneic cells, the opportunity to do so in H-2 incompatible strains lasted for at most a few days after birth (5,451). The viability of the injected antigen is also an essential requirement (450,452), for the persistence of antigen parallels the persistence of tolerance. Thus, abolition of lymphoid cell chimaerism in tolerant mice by injection of third party sensitised cells causes a permanent loss of tolerance even though the inoculated cells are soon destroyed by the host (453). Finally, the ease of tolerance induction is inversely related to the magnitude of the histocompatibility barrier which is to be crossed (454), this most likely reflecting the large number of clones which have to be inactivated at a given stage of ontogeny to cause tolerance to "strong" antigens.

Burnet (455) proposed that the failure of animals to respond to "self" antigens was caused by either their failure to gain access to the immune system or by specific inactivation or elimination of potentially reactive clones following an encounter between the immune system and the self antigen early in ontogeny. Neonatally induced tolerance to alloantigens has long been considered as an experimental model for self tolerance and therefore as a means of testing Burnet's theories. The ability of tolerant mice to retain skin grafts indefinitely, although consistent with a clonal elimination/inactivation theory, is by no means sufficient evidence of the absence of immune reactivity. Thus both neoplastic (456) and normal (457,458) tissue grafts can survive in a normally reactive host following administration of antibody, and there is now clear evidence that many kinds of syngeneic tumours bearing tumour specific antigens are protected from immune destruction by serum blocking factors (459). Furthermore, there are now many

experimental models in which the induction of specific immunological unresponsiveness* by antigen pretreatment has been found not to be due to the elimination of reactive cells but rather to the intervention of an active suppressor mechanism.

A rather unique situation is that of unresponsiveness to pneumococcal polysaccharides. Injection of small quantities of type 3 pneumococcal polysaccharide into adult mice induces a thymus-dependent IgM response (460) whereas injection of a single large dose of the antigen leads to long term paralysis (461). However, in this state of paralysis in which serum haemagglutinating antibodies cannot be detected, plaque-forming cells are clearly apparent in the spleen and antigen can be detected in the serum. Furthermore, when serum from such paralysed mice is treated with high salt concentrations, free antibody appears. Apparently in animals immunised with high doses of antigen, the resulting antibodies are neutralised by the persisting poorly metabolised antigen. Only when much higher doses of antigen are given is there any central suppression of the plaque-forming cells (461). Even then the central suppression is rapidly reversed when spleen cells from such high dose unresponsive animals are transferred to irradiated recipients, responsiveness being elicited to an antigenic challenge given after an interval of only 2 days (462).

This short lived unresponsiveness of tolerant B cells after their removal to an antigen-free environment is supported by the detailed studies of Weigle and colleagues into unresponsiveness in mice induced by the thymus-dependent antigen, human gamma globulin

* In the discussion that follows and in the rest of this chapter, the word tolerance will be reserved for those situations where there is good evidence that the unresponsive state is due to an irreversible inactivation or elimination of reactive cells rather than to an active suppression of potentially reactive cells.

(463). In contrast to Howard's findings with pneumococcal polysaccharide, no plaque-forming cells are detectable at any time during tolerance induction with HGG. Furthermore, when thymus or bone-marrow cells are removed from mice at various times after the administration of a tolerogenic dose (2.5 mg) of de-aggregated HGG and injected with normal bone-marrow or thymus cells respectively into an irradiated host, it is found that within 2 days of treatment thymocytes can no longer cooperate with bone-marrow cells, whereas a similar non-cooperative ability in bone-marrow cells is not detectable for 8 days (464). In addition, whereas the unresponsive state of thymocytes persists for about 120 days, bone-marrow cells have regained full responsiveness by 50 days. The interesting point about this model is that unresponsiveness measured in the animal follows exactly the unresponsiveness of its thymus cells and bears no relation to the ability of its bone-marrow cells to react. In other words, unresponsiveness in T cells can be a sufficient pre-requisite for unresponsiveness in the whole animal, at least as far as thymus-dependent protein antigens are concerned.

Further work in this system has indicated that the degree of B cell paralysis detected may be largely dependent on the technique used to measure it. Thus, Kaplan and Cinader found that whereas a normal PFC technique, as used by Weigle et al (464), failed to reveal responsiveness in bone-marrow or spleen cells when transferred to an irradiated recipient, the use of an antigen elimination method or a more sensitive PFC technique showed that they were still partially responsive (465). The extreme difficulty of inducing unresponsiveness in B cells has been underlined by the studies of Mitchison, who found that educated thymocytes could still restore responsiveness to mice chronically paralysed by injections of BSA unless the amount of antigen given was 100 - 1,000 times greater than that needed to

suppress antibody formation in the absence of transferred thymocytes (466). Similarly, induction of unresponsiveness to haptenic determinants is extremely difficult to achieve (467).

One possible explanation of the apparently high susceptibility of T cells to "tolerance" induction as assayed by transfer experiments (464) is that suppressor cells are induced which inhibit the responsiveness of the normal B cells in the reconstituted animal. However, evidence against this happening in the HGG system is the extreme rapidity of the onset of unresponsiveness and the absence of any detectable response during the period of induction. More convincing evidence against suppressor cells in this situation is the finding (468) that induction of unresponsiveness occurs perfectly well in thymectomised mice (in contrast to the situation when SRBC is the antigen - see below). Furthermore, spleen cells from mice unresponsive to HGG do not inhibit the responsiveness of normal spleen cells when transferred to irradiated mice (469). As pointed out above, the unresponsiveness in T cells is long-lasting, and is therefore presumably maintained by the extremely small quantities of protein antigen likely to be present in these animals long after injection. A similar form of unresponsiveness was described by Mitchison (470), who found that either very low or very high quantities of protein antigen (BSA) could cause unresponsiveness, whereas intermediate doses lead to immunity. It now appears that a likely interpretation of these experiments is that low doses of antigen can paralyse T cells whereas very high doses of antigen cause paralysis of T and B cells.

Although antigens may be able to interact with cells directly and cause them to become refractory to a subsequent antigen challenge, as seems to be the case for neonatally induced tolerance to allogeneic cells or for unresponsiveness to at least some "self" components (see below), there are now many reports suggesting that either antibody or

suppressor cells play an important facilitating role in some forms of specific unresponsiveness.

The most well documented example of antibody-mediated suppression is that of immunological enhancement. Although originally the phenomenon was only clearly demonstrable with tumour allografts, two groups of workers (457, 458) have found that F_1 kidneys are protected to a remarkable degree if hyperimmune serum is administered to parental recipients for a short period after grafting. The presence of potentially reactive cells in these rats was shown by the ability of lymphocytes from enhanced rats to induce GVH reactions in F_1 hosts (471).

There are other systems where antibody seems to be involved in suppression. Ryder and Schwartz have described a remarkable series of experiments where the minute amounts of anti-SRBC antibody remaining in mice more than 6 weeks after injection prevented the animals from responding to antigen challenge (472). Spleen cells were found to be normally responsive when transferred into irradiated recipients. By contrast, whereas antigen-treated peritoneal cells from normal mice could induce an antibody response when injected into normal hosts, the corresponding cells from tolerant mice could not do so. The implication is that macrophages were important in maintaining the unresponsive state - possibly traces of antibody retained on their surfaces presented antigen in a "tolerogenic" manner. In a rather similar system Terman et al (473) have reported that injection of neonatal mice with antibodies to bovine serum albumen resulted in their having diminished responsiveness to this antigen in later life. Unresponsiveness could also be produced by injection of the antigen into neonatal mice, serum recovered from these partially tolerant mice in later life being able to act in a similar immunosuppressive manner to immune serum when re-injected into neonates. Interestingly, heat treatment of spleen cells from antigen-treated unresponsive mice

resulted in the liberation of antigen-antibody complexes. In view of the findings of Ryder and Schwartz (472) it may well have been that these were released from macrophage surfaces.

A strong suggestion that antibody can be a mediator of unresponsive states has come from the in vitro studies of Diener and Feldmann (474). They have shown that treatment of B cells with high concentrations of polymerised flagellin can cause unresponsiveness to subsequent challenge with the specific antigen. By contrast, unresponsiveness cannot be induced to non-polymerised antigens in this way. However, when small amounts of antibody are added to the incubation mixture unresponsiveness can be induced to a variety of "monomeric" antigens. Diener and Feldmann have postulated that an important event in this in vitro model of suppression is the formation of a matrix of antigen on cell surfaces which cross-links and immobilises the receptors and effectively inactivates the cells. Although this model is very attractive it is not clear yet how relevant it is to in vivo situations.

Recent studies in a number of systems have provided evidence that some forms of unresponsiveness may be mediated by suppressor cells. One situation where suppressor cells are apparently operative is in mice which have been made tolerant to sheep red blood cells. Gershon and Kondo (475) found that the induction of unresponsiveness in thymectomised, irradiated, bone-marrow reconstituted mice was facilitated by the addition of thymocytes to the system. The unresponsiveness induced in the presence of thymocytes was not easily broken by transfer of further normal thymocytes and subsequent studies showed that spleen cells from these mice, when transferred to secondary irradiated mice, would specifically inhibit the ability of normal

thymus and bone-marrow cells to cooperate (476). Circumstantial evidence implicated the T cell as the suppressor. A role for suppressor cells in mediating unresponsiveness to SRBC has also been suggested by McCullagh's work. He showed that not only were normal cells unable to break the unresponsive state but that they themselves became unresponsive after 3 days residence in the tolerant host (477). Later experiments demonstrated that thoracic duct or spleen cells, but not bone-marrow cells, could transfer the unresponsiveness to normal rats or (more efficiently) to rats that had been subjected to thoracic duct drainage (478).

Another system where suppressor cells seem to be acting is that of mice suffering from allotype suppression. Mice born as a result of mating a normal SJL male mouse with a Balb/c female pre-immunised against an immunoglobulin allotype of SJL mice show a marked and chronic reduction in ability to synthesise immunoglobulin bearing the target allotype (479). When spleen but not bone-marrow cells were transferred to irradiated mice the ability of the hosts to synthesise immunoglobulin containing the target allotype was frequently inhibited, and similarly when normal cells and cells from allotype suppressed mice were transferred together, allotype synthesis by the former cells was gradually reduced over a period of weeks (480). These effects could be produced in vitro and preliminary evidence from culture studies suggests that both T cells and a soluble factor are involved in the suppressor mechanism (481).

In each of these various systems a feature of the unresponsive state is the demonstrable presence of reactive cells, and in the great majority of cases the unresponsiveness induced is only partial, there being a small but measurable residual reactivity to the tolerising antigen. These various models would therefore appear to be inadequate as a means of protecting the body against autoimmune

reactions where the smallest levels of reactivity could have serious consequences. A more reasonable view is that the active suppressor mechanisms described here serve to regulate the normal immune response. Such regulation is essential and undoubtedly complex, and many different suppressor mechanisms are likely to be needed to deal with the diversity of immune reactions. It is to be anticipated that further analysis of normal immune responses will reveal suppressor mechanisms, which at the present time are only clearly demonstrable in rather artificial situations, to be an integral part of antigen-induced responses.

By contrast to the systems described above, there has for many years been a body of evidence indicating that neonatally induced tolerance to transplantation antigens is not maintained by active suppression. Among the points favouring this concept are:

(a) Attempts to demonstrate GVH reactivity against the donor strain with cells from tolerant animals produced completely negative results (482, 483).

(b) Similarly, mixed lymphocyte cultures with cells from tolerant rats failed to reveal significant reactivity (331, 484).

(c) Tolerance is most easily induced against weak histocompatibility antigens against which antibody formation is not usually detectable (454).

(d) Tolerance can be broken with both sensitised and normal cells (253, 450).

However, in 1971 Hellström et al (393) presented results which suggested that CBA and A strain mice made tolerant at birth by injection of 50 million F_1 spleen cells contained not only cells cytotoxic to fibroblasts of the tolerising strain but also serum factors able to block this reactivity. In order to investigate the validity of these claims a detailed study of the in vitro reactivity of cells from

tolerant mice of the same strain used by Hellström et al was undertaken. The results of these studies are described in the following sections.

1. In vivo studies of the ease of induction of tolerance.

In order to investigate the claim of Hellström et al (393) that tolerance is mediated by blocking factors most of the work reported here was done in the same strain combination as used by them, i.e., CBA mice tolerant to A.

However, because of the relatively weak MLC reactivity between CBA and A cells attempts were made to induce tolerance in CBA mice to other strains against which there are stronger mixed lymphocyte responses in vitro. In this way a greater degree of resolution would be possible in the in vitro testing of reactivity in tolerant cells, and information from different strain combinations would be a useful supplement to the studies in the "Hellström" combination.

When attempts to induce tolerance in other combinations were made, it was found to be possible only with great difficulty. It must be pointed out that the various experiments were not done under strictly controlled conditions, i.e. injection of litter-mates with the different doses or strains of cells. The results, therefore, are only suggestive and detailed interpretation are not possible. Nevertheless some general conclusions can be made.

The first is that of the 4 strain combinations investigated tolerance induction is by far the easiest in the A→CBA combination. The 89% success rate (Table 36) is in fact an underestimate since a large number of the observed failures can be attributed to non-specific problems e.g., graft slippage, infection, scratching and chewing by recipients and also to occasional technical errors at the injection stage (see below for further discussion on these points). Hellström et al (393) claimed that 50 million (CBA x A) F_1 spleen cells were able to

TABLE 36.

F ₁ cells injected			CBA → A		A → CBA		C57B1 → CBA		DBA/2 → CBA	
Number (million)	Type	Route	No. of mice grafted	% tolerant	No. of mice grafted	% tolerant	No. of mice grafted	% tolerant	No. of mice grafted	% tolerant
50	Sp	ip	78	29%	278	89%	9	0%	6	0%
10-12	Sp	iv	11	45%	12	100%	9	11%	12	0%
15-20	Sp	iv	ND		ND		5	40%	5	20%
18-20	BM	iv	ND		ND		32	53%	17	29%
Difficulty of tolerance induction			++		+		+++		++++	

All newborn mice injected were less than 24 hrs old. The first column shows the total number of F₁ hybrid cells injected (viabilities were 60-75% for cells injected ip; 75-85% for cells given iv), the second column the origin of cells (Sp = spleen; BM = bone-marrow), and the third column the route of injection. For each strain combination the number of mice surviving to be grafted and the % of mice retaining 100% grafts for at least 6 weeks is shown.

induce complete tolerance in CBA mice to A strain antigens and the results reported here completely verify that claim. Hellström et al also claimed that 50 million (CBA x A) F_1 spleen cells were able to induce complete tolerance in A strain mice to CBA antigens. The same experiment performed in this laboratory showed that out of 78 A strain mice so treated less than 1 in 3 became fully tolerant (Table 36). Unless there is a significant difference between the immunological reactivities of the A strain mice in this laboratory and in the laboratories of the Clinical Research Centre in London where the mice used by Hellström et al were made tolerant, it must be concluded that these workers seriously underestimated the efficacy of their tolerance-inducing treatment in A strain mice. Furthermore, at the time when these experiments were performed the CBA mice at the National Institute for Medical Research, London were going through a period of hyper-reactivity (485) and it is possible that the normally tolerogenic dose of 50 million (CBA x A) F_1 cells was, at that time, not sufficient for tolerance induction. Whether the treatment given to the mice tested by Hellström et al was sufficient to induce complete tolerance is a crucial point, since the mice used for in vitro tests were not themselves grafted to check the existence of a tolerant state. Furthermore, it has been shown that at least some mice given doses of cells at birth insufficient to induce complete tolerance have cells which are cytotoxic in the micro-cytotoxic assay, and serum factors which can inhibit killing in this assay (485).

The remaining results in Table 36 show that induction of tolerance in CBA mice to either C57B1 or DBA/2 antigens is even more difficult than in the CBA $\rightarrow$ A system. Indeed, even when 18-20 million F_1 bone-marrow cells are injected intravenously tolerance induction is far from completely successful.

TABLE 37. H-2 and M locus antigens against which tolerance induction was attempted.

Recipient strain	Donor Strain	K-end antigens		D-end antigens		Public SD H-2K + H-2D	M locus antigen
		Private SD H-2K	MLC	MLC	Private SD H-2D		
CBA	A	-	-	d	4	6,10,13,14,27,28,29,35,36,41,42,43,44,	3 (moderate)
CBA	C57B1	33	b	b	2	6,14,27,28,29,39,46,53,54,56,	2 (weak)
CBA	DEA/2	31	d	d	4	6,10,13,14,27,28,29,34,35,36,41,42,43,44,46,	1 (strong)
A	CBA	-	-	k	32	-	prob.2 (weak) ⁺

The table shows the antigens possessed by the donor F_1 cells but absent from the recipient.

Information on SD antigens from reference 487.

Information on M antigens from reference 488.

+ Although the M locus antigen of CBA/Caf mice used in these studies has not been determined the weak positive responses found in CBA/Caf v. (CBA/Caf x C3H/He) F_1 cultures show that it does not possess the C3H/He M antigen which is M^3 . Furthermore, no response is found in A/Jax v. (CBA/Caf x A/Jax) F_1 cultures showing that the CBA/Caf strain does not possess the M^1 or M^4 antigens which are both strongly stimulatory. It thus seems most likely that it possesses the M^2 antigen, which is the M locus allele of CBA/H mice. (The data quoted for the MLCs with CBA/Caf mice are unpublished observations of the author).

Nevertheless, sufficient tolerant mice could be produced in these combinations for the necessary *in vitro* tests.

The question arises as to what factors control the induction of tolerance in CBA mice to the three strains of cells used. A summary of the known differences in LD and SD antigens between the strains tested for tolerance induction is shown in Table 37.

In the A→CBA combination, where tolerance induction is relatively easy, there are 14 SD differences, whereas in the much more difficult combination C57B1→CBA there are 12 SD differences. Furthermore, 5 of the SD differences are shared by the two combinations, so that one would have to postulate that the remaining 7 SD differences in the latter combination present a much stronger histocompatibility barrier than the 9 remaining differences in the former combination in order to account for the tolerance induction data. More convincing evidence that H2 SD antigens do not play a decisive role in tolerance induction comes from an examination of the DBA/2→CBA combination in which tolerance induction is extremely difficult. Here there are 17 SD differences yet only 3 of these differences are not also shared by the A→CBA combination. It seems unlikely that these 3 additional SD differences could account for the very much greater barrier to tolerance induction in the DBA/2→CBA combination than in the A→CBA combination.

A much more consistent picture is obtained when LD antigens are considered. In the A→CBA combination there is no major LD difference, there being only disparity at the minor H2 LD locus. There is probably also only a moderate antigenic barrier at the M locus (see footnote to Table 37). By contrast the C57B1→CBA combination includes a major LD-1 disparity, and the combination DBA/2→CBA includes not only a major LD-1 disparity but probably also a strong M locus disparity.

To confirm that the levels of MLC response between donor and

recipient strains were those expected from the known LD locus data, the results from all experiments in which CBA cells were stimulated with two different antigens were used to calculate the relative immunogenicity of each strain to CBA cells in MLC (Table 38). The order of MLC stimulating ability was $A \ll C57B1 < DBA/2$. This correlates perfectly with the relative ease of tolerance induction

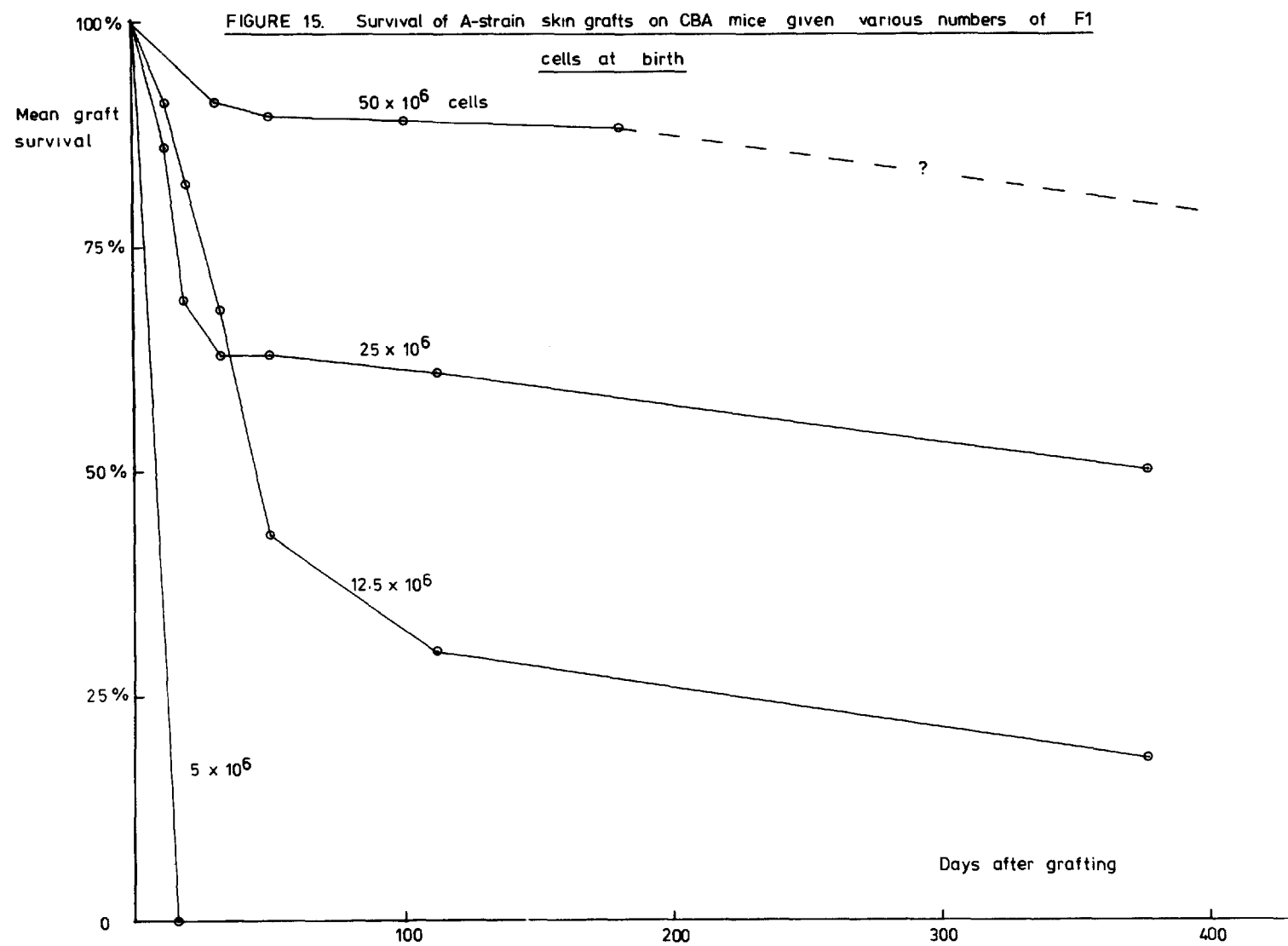
TABLE 38.

Relative MLC responses of CBA cells to the tolerogenic strains of antigens.

Antigens compared	C57B1/ _A	DBA/2/ _A	DBA/2/ _{C57B1}
No. of expts.	2	10	4
Mean ratio of indices	4.28	5.60	1.37

From the results of every experiment in which 2-6 month old CBA mice were stimulated in MLC by 2 different strains of stimulator cells the ratio of indices $I_x - 1 / I_y - 1$ (where x and y stimulator cells were either both parental or both F_1) was calculated. The geometric mean of the ratios is shown above.

to these antigens in newborn CBA mice. The results described in this section thus provide circumstantial evidence that the ease of tolerance induction in neonatal mice is determined by the degree of LD-antigen disparity between the strains involved. A finding which seems at first sight to be contradictory to this hypothesis is the moderate difficulty of tolerance induction in the $CBA \rightarrow A$ direction when 2/2 MLC experiments showed no significant response of A strain lymph node cells to CBA antigen. This apparent discrepancy, however, could be caused by differences in immunological maturity of the different strains of mice at birth. On the other hand, experiments performed with the same colonies of mice several years ago (451) showed that tolerance induction in newborn A strain mice to CBA antigens could be



induced very effectively with only 5-6 million F_1 spleen cells given iv, contrasting with the partial success obtained in the present experiments with 10-12 million F_1 spleen cells. This suggests that other factors such as maternal diet or infection can affect the ease of tolerance induction in newborn mice, although as the results for this combination described here are based on a single experiment further investigation is necessary to confirm the apparent change in susceptibility to tolerance induction.

2. Tolerance induction in the A $\rightarrow$ CBA combination.

As already discussed in section 1, tolerance induction in this combination is relatively easy. Figure 15 shows the mean % survival of A strain skin grafts on CBA mice given varying numbers of (CBA x A) F_1 cells at birth. The curve for mice given 50 million cells at birth is based on the data for 278 mice treated in 26 separate experiments. The curves for mice given lower doses of F_1 cells at birth are based on a single experiment in which groups of 8-10 mice given the various doses of cells at birth were grafted with A strain skin.

With mice given 50 million F_1 cells, virtually all the observed rejections occurred rapidly within a few days of unbandaging, the remaining 90% of mice showing long term tolerance, with a very rare incidence of graft rejection. When the mice are older than about 8 months, deterioration of some grafts - hair loss and contraction - is observed. Since defects of the immune system, in particular autoimmunity become increasingly common in aging animals (431), graft rejection due to loss of tolerance is a possibility. However, since the deterioration of grafts in old mice was, at least in most cases, accompanied by mite infections which can cause damage to the host skin, deterioration of some grafts in old mice was probably unrelated

to their allogeneic nature. Furthermore, in old mice hair loss from host skin frequently occurred and graft deterioration may have been merely a reflection of the aging of host tissues, and perhaps also of the grafted skin.

The early graft rejections which are observed in about 10% of mice given 50 million F_1 cells at birth could be due to one of 3 reasons: (i) failure of 50 million F_1 cells to induce complete tolerance in some recipients, (ii) technical errors at the time of injection, or (iii) failure of the test graft to 'take' - grafts can be displaced from the graft bed, succumb to infection, fail to heal-in properly before unbandaging, or be damaged by scratching or chewing after unbandaging. Rejection due to the first two reasons would be complete, except in a few cases where partial tolerance may have been induced, whereas breakdowns due to non-immunological reasons would in most cases be expected to be only partial. To investigate this further, 31 mice showing either complete or partial rejection of test grafts were regrafted on the opposite flank. The survival of these second grafts was observed for at least 2 months with the following results:

First graft	Second graft	
	accepted	rejected
Complete breakdown	3	13
Partial breakdown	14	1

The majority of mice whose first grafts broke down also rejected second grafts, whereas the majority of mice whose first grafts showed only partial breakdown at 20 days accepted second grafts indefinitely. Indeed, more than half the mice (17/31) in this limited study with grafts that had either completely or partially broken down were in fact

tolerant, and therefore when account is taken of the other source of technical failure (errors at the time of injection) it is likely that at least 95% of newborn CBA mice given 50 million F_1 cells at birth are made fully tolerant to A strain antigens. The small percentage of mice for which this treatment is insufficient are presumably too immunologically mature at the time of injection to be made tolerant.

In the experiment shown in Figure 15, where mice were given lower doses of F_1 cells at birth, those mice given 25 million cells showed much higher numbers of early breakdowns than was found in mice given 50 million cells. However, those grafts that survived for longer than 20 days rarely showed any subsequent sign of breakdown. Mice bearing these grafts can therefore be regarded as fully tolerant. By contrast, mice given $12\frac{1}{2}$ million cells at birth not only showed a high rate of early breakdowns but also a phase between 20 and 110 days after grafting in which many (5/10) grafts showed a chronic deterioration. Such mice can certainly not be considered fully tolerant, and in this thesis the term partially tolerant is used to describe them. The protracted breakdown of these grafts suggests an active suppression of the immune system, and the finding that serum from 4/8 ungrafted mice from the same group was able to block killing by immune cells in the microcytotoxic assay (486) implies that serum-borne inhibitory factors may have been responsible for the prolonged survival of grafts on such mice.

The results of all experiments in which newborn CBA mice were given lower doses of cells than are necessary for the induction of full tolerance are shown in Table 39.

About 40% of mice given 25 million F_1 cells at birth appear to be fully tolerant as judged by their indefinite retention of perfect skin grafts. By contrast only 3/48 mice given $12\frac{1}{2}$ million F_1 cells

TABLE 39. Graft survival in CBA mice given less than a full tolerogenic dose of (CBA x A)F₁ cells at birth. .184

No. of cells given (million)	No. of mice grafted	Approx. MST	Number of mice with 50% rejection at:			No. of fully tolerant mice
			20 days	20-100 days	100 days	
<u>Experiment 1.</u>						
25	3 male 5 female	8 mo.	3	0	1	4
12½	4 male 6 female	47 d. 47 d.	2	5	2	1
5	5 male 5 female	12 d.	10	0	0	0
<u>Experiment 2.</u>						
25	5 male 5 female	36 d.	4	3	0	3
12½	6 male 4 female	19 d. 12 d.	7	3	0	0
5	4 male 6 female	11 d.	10	0	0	0
<u>Experiment 3.</u>						
12½	6 male	110 d.	1	1	4	0
12½	7 female	14 d.	5	1	0	1
<u>Experiment 4.</u>						
12½	8 male	13 d.	5	3	0	0
12½	7 female	13 d.	5	1	0	1

Mice were grafted when 5-7 weeks old (expts. 1, 3 & 4) or when 6 months old (expt. 2). The MST (time at which the mean graft survival fell to 50%) and the times at which individual grafts showed less than 50% survival are given for each group. In the last column the number of mice retaining perfect grafts indefinitely (at least 120 days post-grafting) is shown.

behaved in this manner. As mentioned above many mice in this latter class show a chronic graft rejection which usually takes place between 20 and 100 days after grafting although in some cases it continues beyond 100 days. A total of 20 out of 48 mice given $12\frac{1}{2}$ million F_1 cells at birth subsequently showed this chronic form of graft rejection. The phenomenon was also observed occasionally, but less frequently, in mice given 25 million F_1 cells at birth (4/18); it occurred very rarely in mice given 50 million F_1 cells (11/207). Mice given 5 million F_1 cells at birth showed no sign of either full or partial tolerance and in fact may be slightly sensitised as shown by their rapid graft rejection and the presence of cytotoxic cells in some of these animals (486).

3. The MLC reactivity of cells from mice shown to be fully tolerant to alloantigens by skin grafting.

(a) Tests on young mice.

A detailed study of the MLC reactivity of tolerant chimaeric mice, in particular the A-CBA combination used by Hellström et al (393), is described here. Except in 2 experiments (see below) all tolerant mice used in these tests were shown to be tolerant by the retention of perfect allogeneic tail skin grafts for at least 3 weeks prior to testing, and in most cases for very much longer. Evidence that such mice have a high level of cellular chimaerism has been presented elsewhere (486).

Table 40 shows the results of a typical MLC experiment in which the reactivity of cells from two CBA mice tolerant to A strain antigens was tested. At no day on which MLC reactivity was assayed was there any sign of reactivity in the tolerant cells to the specific tolerising antigen. Indeed, cells from mouse T2 had indices significantly less than unity on days 2-3 and 4-5 ($P=0.03$. in both

TABLE 40. MLC reactivity of cells from tolerant mice I. Grafted.

	CBA cell Background	Stimulation by (CBAXA) F_1 cells	I_A	I_{C57}	I_{C3H}	% CBA cell survival
<u>Day 2-3</u>						
T1	270 $\pm$ 20	-50 $\pm$ 60	0.96 $\pm$ 0.04	1.87 $\pm$ 0.09	0.95 $\pm$ 0.05	37 $\pm$ 3
T2	270 $\pm$ 10	-140 $\pm$ 60	0.89 $\pm$ 0.04	2.28 $\pm$ 0.11	0.81 $\pm$ 0.04	37 $\pm$ 3
N	680 $\pm$ 60	440 $\pm$ 140	1.30 $\pm$ 0.10	2.31 $\pm$ 0.12	1.12 $\pm$ 0.08	35 $\pm$ 2
<u>Day 3-4</u>						
T1	340 $\pm$ 30	100 $\pm$ 170	1.07 $\pm$ 0.12	3.00 $\pm$ 0.24	1.05 $\pm$ 0.09	19 $\pm$ 3
T2	340 $\pm$ 20	30 $\pm$ 190	1.03 $\pm$ 0.13	3.96 $\pm$ 0.29	1.06 $\pm$ 0.14	19 $\pm$ 3
N	730 $\pm$ 70	1,330 $\pm$ 220	1.79 $\pm$ 0.18	4.24 $\pm$ 0.32	1.46 $\pm$ 0.10	16 $\pm$ 3
<u>Day 4-5</u>						
T1	250 $\pm$ 20	-110 $\pm$ 120	0.93 $\pm$ 0.08	4.07 $\pm$ 0.23	0.75 $\pm$ 0.10	11 $\pm$ 1
T2	290 $\pm$ 20	-500 $\pm$ 170	0.67 $\pm$ 0.10	5.31 $\pm$ 0.27	0.99 $\pm$ 0.11	9 $\pm$ 1
N	760 $\pm$ 40	2,360 $\pm$ 160	2.34 $\pm$ 0.13	5.96 $\pm$ 0.31	2.06 $\pm$ 0.16	13 $\pm$ 1

Lymph node cells from two individual tolerant CBA mice bearing perfect A-strain grafts for 30 days and from age and sex matched normal CBA mice were cultured in tubes either alone or mixed with (CBA x A) F_1 , (CBA x C57Bl) F_1 or (CBA x C3H) F_1 spleen cells. Cultures were labelled after 48, 72 and 96 hrs with H-3 thymidine for 16 hrs. Figures for DNA synthesis are cp5m/100 microl. of culture.
I = Index with appropriate F_1 cells).

cases) to (CBA x A) F_1 cells. By contrast, the cells from tolerant mice showed good reactivity to third party C57Bl antigens. However, their reactivity was not as strong as that of cells from normal mice. Indeed, when their reactivities to a second third-party strain, C3H, were measured no reactivity at all could be detected. CBA and C3H mice are H2 identical (H-2^k) and the MLC response of CBA cells to (CBA x C3H) F_1 cells is at best very weak. But the difference between the reactivities of normal and tolerant (to A) CBA mice is clearly apparent by day 4-5 of culture. The subnormal reactivity of tolerant cells to third party strains is discussed in greater detail below.

The work of Hellström et al (393), in which the presence of reactive cells in tolerant mice was suggested, was done on mice which had not been grafted. In a second time-course MLC experiment the ability of cells from ungrafted tolerant mice to react in MLC was studied. The results, shown in Table 41, were in complete agreement with those of Table 40. from grafted mice, showing that there was no detectable MLC responsiveness at any of the MLC assay points. This experiment was repeated on a separate occasion with 10 week old ungrafted tolerant mice: the result was the same.

As mentioned in Section 1 of this chapter, tolerance induction in the A $\rightarrow$ CBA combination is relatively easy. MLC tests were therefore performed on the cells of CBA mice made tolerant to C57Bl or DBA/2 antigens where the barriers to tolerance induction are much more formidable (Section 1).

The results of two experiments where C57Bl was the tolerogenic strain are shown in Table 42. In the C57Bl $\rightarrow$ CBA combination, even when large numbers of F_1 cells are given intravenously just after birth, tolerance induction is only about 50% successful (see Table 36).

TABLE 42. MLC reactivity of cells from tolerant mice III.

Mouse	State of skin graft	CBA cell background	I _{C57B1}	I _{DBA/2}	% CBA cell survival
<u>Experiment 1.</u>					
T1	100%	6,690 $\pm$ 250	0.80 $\pm$ 0.04	4.72 $\pm$ 0.19	38 $\pm$ 3
T2	100%	6,420 $\pm$ 300	1.05 $\pm$ 0.07	5.17 $\pm$ 0.30	ND
N	-	8,590 $\pm$ 350	3.29 $\pm$ 0.14	5.70 $\pm$ 0.29	33 $\pm$ 2
<u>Experiment 2.</u>					
T3	100%	13,250 $\pm$ 500	1.15 $\pm$ 0.07	5.27 $\pm$ 0.19	34 $\pm$ 2
T4	25%	16,000 $\pm$ 600	4.78 $\pm$ 0.30	5.67 $\pm$ 0.40	43 $\pm$ 3
N	-	13,750 $\pm$ 150	4.77 $\pm$ 0.16	5.64 $\pm$ 0.22	40 $\pm$ 3

Lymph node cells from CBA mice with perfect or imperfect C57B1 skin grafts were cultured in MLC with (CBA x C57B1) F_1 and (CBA x DBA/2) F_1 lymph node cells. Cells from normal mice of same age and sex as tolerant mice (except in the case of T4) were used as controls. In experiment 1 cultures were labelled after 90 hrs with H-3 thymidine for 8 hrs; in experiment 2 cultures were labelled after 75 hrs for 14 hrs. Figures for DNA synthesis are cp5m/100 microl. of culture.

TABLE 41. MLC reactivity of cells from tolerant mice II. Ungrafted.

	CBA cell background	Stimulation by (CBA x A) F_1 cells	Index	% CBA cell survival
Day 2-3				
T	520 $\pm$ 50	20 $\pm$ 80	1.02 $\pm$ 0.09	40 $\pm$ 1
N	470 $\pm$ 25	880 $\pm$ 80	2.05 $\pm$ 0.17	48 $\pm$ 2
Day 3-4				
T	770 $\pm$ 70	-30 $\pm$ 90	0.98 $\pm$ 0.08	25 $\pm$ 3
N	850 $\pm$ 110	1,610 $\pm$ 250	2.36 $\pm$ 0.25	32 $\pm$ 1
Day 4-5				
T	960 $\pm$ 35	-240 $\pm$ 220	0.82 $\pm$ 0.16	15 $\pm$ 1
N	1,030 $\pm$ 60	1,870 $\pm$ 400	2.36 $\pm$ 0.31	23 $\pm$ 1

Lymph node cells from a pool of two 14 week old CBA mice injected at birth with 50 million (CBA x A) F_1 cells and from age and sex matched normal CBA mice were cultured in MLC with (CBA x A) F_1 cells. Cultures were labelled after 48, 72 and 96 hrs with H-3 thymidine for 16 hrs. Figures for DNA synthesis are cp10m/100 microl. of culture.

Mice T1 and T3 received 19 million (CBA x 57B1) F_1 bone-marrow cells intravenously at birth. Their retention of a perfect skin graft for 7 weeks and 5 weeks respectively showed tolerance induction had been successful, and MLC testing showed no reactivity in vitro to C57B1 antigens. Mouse T2 received 12.5 million F_1 bone-marrow cells iv at birth. Once again it had a perfect skin graft for 7 weeks and its cells were non-reactive in MLC. By contrast, mouse T4 was given 13 million F_1 spleen cells iv at birth. The skin graft on this mouse was initially perfect, but over a period of 18 weeks a chronic deterioration occurred, and, at the time of testing, the percentage epithelial survival was only about 25%. Such a pattern of graft breakdown is common in mice given an insufficient dosage of cells at birth to induce complete tolerance (see Section 2 of this chapter).

TABLE 43. MLC reactivity of cells from tolerant mice. IV.

Cells tested	Tolerant to	Syngeneic CBA background	I _{C57B1}	I _{DBA/2}	Stimulation with PHA
<u>Experiment 1.</u>					
T LN	DBA/2	3,240 $\pm$ 270	ND	0.52 $\pm$ 0.05	7,550 $\pm$ 430
N LN	-	1,480 $\pm$ 230	ND	12.3 $\pm$ 2.2	6,830 $\pm$ 880
T Sp	DBA/2	430 $\pm$ 80	ND	1.0 $\pm$ 0.3	ND
N Sp	-	1,190 $\pm$ 240	ND	4.6 $\pm$ 1.0	ND
<u>Experiment 2.</u>					
T LN	DBA/2	10,340 $\pm$ 320	3.36 $\pm$ 0.12	0.87 $\pm$ 0.04	ND
N LN	-	9,820 $\pm$ 540	5.06 $\pm$ 0.29	5.88 $\pm$ 0.33	ND
<u>Experiment 3.</u>					
T Sp	C57B1	11,440 $\pm$ 360	0.90 $\pm$ 0.04	2.79 $\pm$ 0.09	ND
N Sp	-	18,240 $\pm$ 440	2.49 $\pm$ 0.07	2.84 $\pm$ 0.13	ND

Spleen and/or lymph node cell suspensions from pools of two tolerant mice and from age/sex matched normal mice were cultured with irradiated syngeneic and semi-allogeneic (expts. 1 and 2) or allogeneic (expt. 3) spleen cells. In experiment 1 cells were also cultured with PHA. In experiment 1 cultures were labelled with H-3 thymidine after 96 hrs for a further 23 hrs; in expts. 2 and 3 after 72 hrs for a further 17 hrs. Tolerant mice had borne perfect allogeneic skin grafts for 8 weeks (expt 1), 5 weeks (expt. 2), and 10 weeks (expt. 3). Figures for DNA synthesis are cp20m/50 microl. of culture. I_x = Index of transformation in MLC with the appropriate stimulator cells.

On MLC testing the cells of this partially tolerant mouse had identical reactivity to those of an age and sex matched control mouse. The fact that the cells of this mouse had a normal MLC reactivity and yet graft rejection was occurring only very slowly strongly suggest that in such partially tolerant mice an active suppressor mechanism is operative.

The experiments in Table 42 clearly demonstrate that CBA mice shown to be fully tolerant to C57B1 by skin grafting do not have cells in their lymph nodes reactive to this strain of antigen in MLC. As found for CBA mice tolerant to A, CBA mice tolerant to C57B1 show good reactivity to third party allo-antigens, although this is somewhat reduced compared to normal.

That the non-reactivity of tolerant cells in MLC is not restricted to lymph node cells is shown in Table 43, which gives data concerning the reactivity of spleen cells from CBA mice tolerant to either C57B1 or DBA/2. There was no detectable MLC reactivity of tolerant spleen cells to the tolerogenic strain of antigen. The experiments in Table 43 also show that CBA mice tolerant to DBA/2 antigens do not respond in MLC.

(b) Tests on old mice.

All MLC tests on fully tolerant mice described so far were performed on mice 2-4 months old. The skin grafting data described in Section 2 show that tolerated grafts usually survive indefinitely; where graft deterioration occurred it was usually associated with infection. Nevertheless some MLC tests were performed on old (6-12 month) mice to examine whether any weakening of tolerance had occurred. These tests are not easy since the ability of cells from aging animals to respond in MLC declines quite sharply, especially in the CBA v. A combination (see Chapter 4 Section 3b). Nevertheless, 7 successful tests were performed on 6-month or 12-month old tolerant

CBA mice bearing perfect A strain skin grafts. Two of these mice did give indexes slightly greater than unity, although much lower than those obtained with cells from normal age/sex matched mice. In view of the increased incidence of autoimmune disease in aging animals these responses may have reflected a breakage of tolerance in the old mice. However, since their skin grafts were still in unblemished condition this explanation seems unlikely and a more tenable hypothesis is that the slight apparent responses in MLC were caused by the technical difficulties involved in measuring the low responsiveness of cells from old mice.

(c) Pooled results.

TABLE 44. Summary of all MLC tests on fully tolerant mice.

	Age of mice	
	2 - 4 months	6 - 12 months
Total number of MLC tests	20	7
No. of tests in which index was significantly ($P < 0.05$) greater than 1.00	0	2
No. of tests in which index was significantly ($P < 0.05$) less than 1.00	7	1
Geometric mean index	0.87	1.149
Significance of differences from 1.00	$p = 0.01$	NS

The tests were performed in the majority of cases (19/27) on individual mice, in the remaining cases (8/27) on cells from pools of two mice. Where tests were assayed on more than one day of MLC the indexes obtained on different days were averaged (geometrically) to give a single value.

The results of all MLC tests performed on fully tolerant mice are summarised in Table 44. Despite the fact that in 2/7 old mice there was a slight apparent responsiveness the mean index for the whole group did not differ significantly from 1.00. Thus even in these old mice there is no evidence of reactive cells in their

peripheral lymphoid tissues. The results for young mice present an even clearer picture. None of the 20 MLC tests on tolerant mice showed any significant response. All cells tested in MLC were routinely washed 2 or 3 times in 15-20 mls of saline (containing 2% FCS) and on one occasion were washed 5 times. Such washing precludes any possibility of serum factors (if they existed) being carried over into culture. The only way by which blocking factors could intervene would be if they were generated in culture. None of the evidence presented above can exclude this possibility. However, there are at least three good reasons for believing that this could not be the case.

(i) The proposal that serum-borne blocking factors mediate neonatally induced tolerance (393) was based upon experiments in which cells from presumed tolerant mice were found to be reactive in vitro. If tolerant mice were able to generate inhibitory factors sufficient to block their own MLC reactivity it would be extremely difficult to explain the apparent reactivity of tolerant cells in an in vitro test (the microcytotoxicity test) performed under conditions similar to those for the MLC tests described here.

(ii) If blocking factors are generated during culture and cause inhibition of the MLC response they are presumably stable to conditions in culture. Yet serum from tolerant mice which has been claimed to contain blocking factors (393) was completely unable to cause any inhibition of the MLC responsiveness of normal cells even when present at concentrations as high as 7% (see section 4 below).

(iii) There is good evidence that in mice made unresponsive in adult life to transplantation antigens the unresponsive state is mediated by an active suppression of potentially reactive cells (Chapter 6). Yet lymph node and spleen cells from such animals are clearly reactive in vitro, showing at most only a slight reduction

TABLE 45. Can tolerant cells suppress the reactivity of normal cells in vitro ?

Section A. Dose-response relationship with normal cells.

Ratio N CBA cells: (CBA x A) _F ₁ cells	Stimulation/ million initial CBA cells (cp5m)	
	Expt. 1	Expt. 2
4:1	ND	5,420 ± 2,020
2:1	ND	6,500 ± 600
1:1	10,480 ± 1,400	10,280 ± 2,200
1:2	15,280 ± 2,940	14,420 ± 970
1:4	18,600 ± 3,750	24,300 ± 6,800

Section B. Response of normal and tolerant cells alone to tolerising antigen.

Experiment	Responding cells	Stimulation in mixed culture	Index	% CBA cell survival
1	T	1,040 ± 680	1.14 ± 0.10	24 ± 2
	N	5,240 ± 770	1.89 ± 0.17	20 ± 2
2	T	-400 ± 820	0.96 ± 0.10	25 ± 2
	N	5,140 ± 1,100	2.01 ± 0.23	11 ± 1

Section C. Response of mixtures of normal and tolerant cells to tolerising antigen.

Experiment	Ratio N:T	Ratio N:(CBAXA) _F ₁	Million CBA cells/culture	Observed stimulation	Expected response of N cells
1	1:1	1:2	0.25	4,040 ± 940	3,910
2	1:1	1:2	0.25	7,520 ± 320	
1	1:3	1:4	0.125	3,540 ± 650	2,670
2	1:3	1:4	0.125	4,560 ± 920	

Section D. Do normal cells respond to tolerant cells ?

Ratio N:T	Index $\frac{\text{(DNA synthesis in mixture of N+T cells)}}{\text{(DNA synthesis in unmixed cultures of N and T cells)}}$
1:1	0.98 ± 0.05
1:3	1.07 ± 0.07

Lymph node cell suspensions were prepared from pools of two tolerant mice bearing perfect A strain skin grafts for 7 weeks (expt. 1) or 5 weeks (expt. 2) and from age/sex matched normal mice. Cells were cultured in both experiments for 72 hrs and then labelled for a further 16 hrs. All figures for DNA synthesis (except in Section A) are cp5m/culture (200 microl.)

in MLC responsiveness compared to the levels of response in control cells: the mechanism which blocks cell reactivity in vivo in this system is certainly not able to block the in vitro reactivity of cells.

(d) Tests for suppressive effects in culture.

Nevertheless, to test directly the possibility that self-suppression of tolerant populations of cells was occurring in culture two experiments were performed in which lymph node cells from tolerant mice were mixed with lymph node cells from normal syngeneic mice and the ability of the mixed population to respond in MLC to the tolerogenic strain of antigen was measured. If the tolerant cells were producing blocking factors in culture then these should also suppress the activity of normal cells. The results of these experiments are shown in Table 45. In both experiments normal and tolerant cells were cultured in MLC both alone and mixed in ratios of 1:1 and 1:3 (normal:tolerant). In the latter cultures the ratio of normal CBA cells to simulating (CBA x A) F_1 cells is not 1:1; therefore, dose-response cultures using various ratios of CBA to F_1 cells (keeping the total cell concentration constant at 1.0 million cells/well) were included in each experiment (Section A in Table 45). The responses induced at the different responder:stimulator ratios in the experiments did not differ significantly. Consequently, the values of the response at each cell ratio were averaged (geometrically) and plotted in a log. v. log. dose-response curve. This is shown in Figure 6' in Chapter 3, Section 10a.

Section B of Table 45 shows that in both experiments the tolerant cells gave no significant response in MLC to F_1 cells bearing antigens of the tolerising strain. By contrast, normal cells gave a

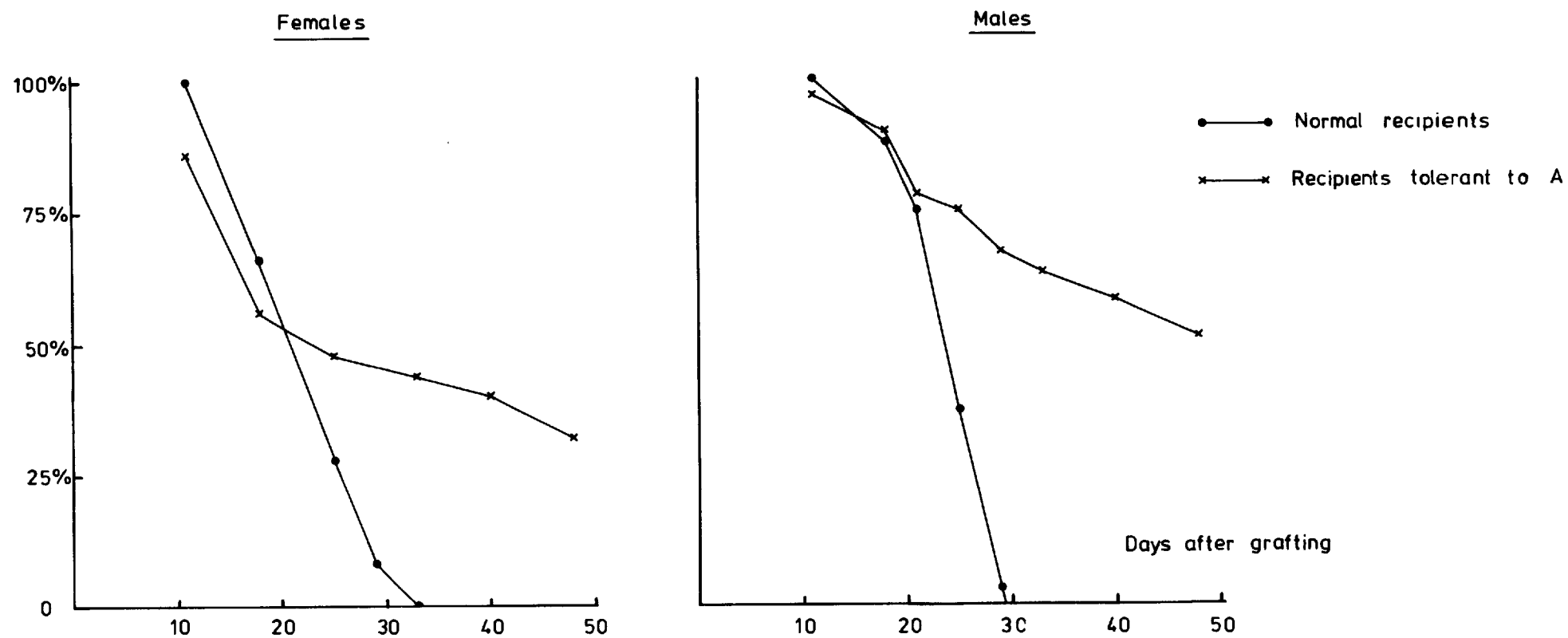
highly significant response to the stimulating cells. Section C of Table 45 shows that even in mixtures where tolerant cells outnumbered normal cells by 3:1 there was no sign of any suppression of the normal cells. It can therefore be concluded that the absence of MLC responsiveness in lymphoid cells from tolerant mice is due to an absence of reactive cells, and not to an active suppression of potentially reactive cells by factors generated in culture.

The mixtures of normal and tolerant lymphocytes in fact showed a somewhat higher responsiveness than could be accounted for from the behaviour of normal cells cultured alone. Since lymphoid cells from these tolerant mice have been shown to be chimaeric (485) it is conceivable that they could stimulate an MLC response in normal lymphocytes. However, control cultures performed to examine this possibility (Section D, Table 45) showed no sign of such a response, indicating that the level of chimaerism provided insufficient stimulator cells to give an MLC response. The most likely explanation of the increased responsiveness in mixtures of normal and tolerant cells is that after specifically induced stimulation of antigen reactive cells in the normal population soluble mediators are liberated. There is in fact growing evidence that such factors can be released following antigen recognition by T cells (see Introduction to Chapter 3). If such substances do participate in mouse MLC responses, then bystander tolerant cells in mixtures of normal and tolerant cells could be caused to transform non-specifically by factors liberated from neighbouring antigen-reactive normal cells.

(e) Level of reactivity to third party antigens.

An interesting finding in mixed lymphocyte cultures with tolerant cells, already alluded to above, is that such cells have diminished reactivity to third party antigens. This is most dramatically demonstrated when the third party antigen is C3H. Normal CBA cells

FIGURE 16. Survival of C3H skin on normal and tolerant CBA mice



reacted weakly but significantly to (CBA x C3H) F_1 cells; cells from CBA animals made tolerant neonatally to A strain antigens had no reactivity at all to (CBA x C3H) F_1 cells (Table 40). A repeat of this experiment gave an identical result. CBA and C3H strains of mice are H-2 identical and the MLC reactivity between these strains is due to M locus differences (382). Since A/Jax and C3H/He mice share the M^3 allele (488), the fact that CBA mice made tolerant to A are also unable to respond in MLC to C3H provides a further demonstration that tolerance can be induced to M locus antigens.

Two experiments were performed to test whether the cross-reactivity shown by the MLC data would cause prolonged survival of C3H skin grafts on CBA mice made tolerant to A, compared to untreated mice. The first experiment suggested that at least in male tolerant mice C3H skin survived longer than on normal controls, but because of "non-specific" graft breakdowns (see Materials and Methods) in some of the subjects, a phenomenon which occurs frequently in this strain combination, the results were not conclusive. In the second experiment, in which 6 male and 6 female mice tolerant to A and bearing A strain skin grafts for 4 weeks and 8 male and 8 female normal CBA mice of the same age were grafted with C3H tail skin, there was a much lower incidence of "non-specific" breakdowns and the few mice in which it occurred were discarded from the analysis. The mean graft survival as a function of time is shown in Figure 16. Graft breakdown in normal and tolerant mice occurred at the same rate for the first 20 days. By day 25, however, an improved survival of C3H skin on mice tolerant to A became apparent, the differences between tolerant and normal recipients being particularly marked in male mice. Thus the inability of cells from CBA mice made tolerant to A to react to C3H-bearing cells in vitro is paralleled by the impaired ability of

such mice to reject C3H skin grafts. This latter observation confirms the previous findings of Billingham and Brent (5).

In other experiments, cells from tolerant CBA mice were cultured in MLC with third party cells from strains other than C3H and their reactivity to these cells compared with that found in cells from age and sex matched normal mice. In 10/10 such tests on lymph node cells from mice tolerant to A, C57Bl or DBA/2, the tolerant cells had lower reactivity⁺ to third party cells than did control cells. Indeed, the mean reactivity of tolerant cells to third party antigens was only 68% of that of control cells, and this figure differed from 100% at the $p = 0.001$ level. This implies that there is a considerable cross-reactivity between MLC antigens in different strains of mice, a finding that parallels the well established cross-reactivity between serologically defined antigens in mouse strains.

(f) Do tolerant mice possess cytotoxic cells ?

The results from mixed lymphocyte cultures described in this section strongly support the idea that lymphoid cell populations do not contain reactive cells. Present evidence concerning the induction of cellular immunity indicates that two stages are involved; an inductive phase, in which lymphocytes recognising LD antigens proliferate, is followed by the production of effector cells which seem to have specificity mainly for SD antigens (377). Effector cells cannot be produced in vitro in the absence of MLC proliferation (see Introduction to Chapter 3). Therefore, if the inductive pathway is non-existent in tolerant animals, as is strongly suggested from .

+ Relative reactivity was calculated as $(I_T - 1) / (I_N - 1)$ where I_T and I_N were the indexes of transformation for tolerant and normal cells cultured with third party cells.

TABLE 46. Are cytotoxic cells present in tolerant mice ?**A. Direct test.**

Effector cells present	Chromium release	Cytotoxicity
N	$3,630 \pm 40$	-
T	$3,660 \pm 30$	$0.2 \pm 0.4\%$
I	$7,330 \pm 140$	$29.2 \pm 1.2\%$

B. Cells presensitised in MLC.

Effector cell	MLC stimulator cell	Target cells	
		EL 4	M
T	C57B1 _x	$2.2 \pm 1.0\%$	$1.1 \pm 1.4\%$
N	C57B1 _x	$73.2 \pm 1.6\%$	$34.9 \pm 1.5\%$
T	DBA/2 _x	$5.4 \pm 1.1\%$	$90.2 \pm 2.7\%$
N	DBA/2 _x	$15.4 \pm 1.5\%$	$96.2 \pm 2.5\%$

In the direct test (A) the cytotoxic activity of spleen cells from one tolerant CBA mouse (bearing a perfect C57B1 skin graft for 14 weeks), from one normal mouse of similar age, and from one normal mouse immunised 5 days previously with 15 million (CBA x A)_F₁ cells were tested by culturing them for 12 hrs with Cr-51 labelled EL4 cells at a 200:1 ratio. In the indirect test (B) spleen cells from two tolerant CBA mice (bearing perfect C57B1 skin grafts for 10 weeks) and from 2 normal age/sex matched CBA mice were cultured in MLC for 4½ days in the presence of irradiated CBA, C57B1 and DBA/2 spleen cells at a responder:stimulator ratio of 2:1. (Some cultures were labelled with H-3 thymidine after 72 hrs and the performance in MLC is shown in experiment 3 in Table 43). Cells were then washed and recultured with Cr-51 labelled EL4 and M cells for 6½ hrs with effector:target cell ratios of 100:1 and 50:1 respectively. % cytotoxicity against these target cells is shown. Figures for Cr-release in Part A are cp10m/100 microl. of culture.

MLC data, then the generation of cytotoxic cells, at least of those types which are active in chromium release tests, should not be possible. Nevertheless, tests were performed on the cells of tolerant mice to examine whether any cytotoxic cells were detectable. Cells were either tested directly, or after attempted MLC sensitisation, and the results are shown in Table 46. The results of these experiments were quite clear cut, there being no significant cytotoxicity in cells taken directly from a tolerant mouse, nor after attempted sensitisation of tolerant cells in vitro prior to testing. When cells from mice tolerant to C57Bl were sensitised to third party antigen in vitro they did show a very small but probably significant killing of EL4 (H-2^b) tumour cells in vitro. This was presumably caused by some non-specific effect of stimulated cells, for the same tolerant cells cultured in MLC with C57Bl stimulator cells, where no stimulation occurred, gave no significant killing. The results of these experiments thus provide no evidence that tolerant mice possess either cells which are directly cytotoxic in a chromium release test, as are found in immunised normal mice, or cells which can differentiate into killer cells when cultured with the tolerising strain of antigen in vitro.

4. Attempts to detect MLC blocking factors in the serum of fully tolerant mice.

As described in Chapter 4 Section 2 the addition of mouse serum to cultures of mouse lymphocytes severely inhibits their ability to synthesise DNA and at the same time can cause an increase in the index of transformation in mixed cultures. Although these non-specific effects can be partially reduced by using a different method of bleeding and by using higher concentrations of FCS in culture the toxic effects of mouse serum were still a serious

problem and in fact limited the maximum concentration of mouse serum that could be used in mixed lymphocyte cultures to about 6-7%. It is therefore conceivable that tolerant serum might contain blocking factors, but because of the necessary dilution of serum in the MLC tests they would not be detectable. However, there are two reasons for believing that they would be detectable. Firstly, sera from mice given a single skin graft or a single injection of 10 million F_1 cells caused a very significant inhibition of the MLC reaction (Chapter 4 Section 4). It is thus quite possible to detect serum-borne inhibitory factors at the low concentrations at which mouse serum can be used in MLC. Secondly, in the experiments of Hellström et al (393), in which serum from presumed tolerant mice was found to block killing in the microcytotoxic test, serum was initially added at a concentration of about 15% to target cells, then after 4-5 minutes most of it removed by dumping. Tests by the author have shown that only about 5-10% of radioactive medium is retained in Falcon Microtest II plates after dumping. Consequently, the final serum concentrations used by these workers must have been about 1-1.5%. Unless the short incubation with higher concentrations is crucial, concentrations of tolerant serum up to 7% would be expected to inhibit MLC reactions if they contained factors similar to those postulated by Hellström et al.

Since in other systems where serum factors have been found to inhibit cellular immunity, the relative concentrations of Ab and Ag in the test mixture have proved crucial if inhibition is to be observed (489), the effects of tolerant serum on the MLC reactivity of normal cells were studied over a wide range of concentrations. An experiment in which several different serum concentrations were used is shown in Table 47. The design of this

TABLE 47. Can serum from tolerant mice inhibit the MLC response of normal cells? I.

203.

Serum concn.	Serum tested	Background in syngeneic cultures	Stimulation in mixed cultures	Index	Response relative to that with N serum
1%	N	4,580 $\pm$ 200	19,310 $\pm$ 310	5.22 $\pm$ 0.24	
	T	4,140 $\pm$ 140	19,080 $\pm$ 600	5.61 $\pm$ 0.24	109 $\pm$ 8% ^{NS}
	H	4,260 $\pm$ 170	10,560 $\pm$ 290	3.48 $\pm$ 0.15	59 $\pm$ 5%***
2%	N	3,270 $\pm$ 170	17,150 $\pm$ 380	6.24 $\pm$ 0.34	
	T	2,820 $\pm$ 130	15,510 $\pm$ 460	6.50 $\pm$ 0.34	105 $\pm$ 9% ^{NS}
	H	2,710 $\pm$ 130	3,260 $\pm$ 230	2.20 $\pm$ 0.12	23 $\pm$ 3%***
4%	N	2,280 $\pm$ 70	16,000 $\pm$ 370	8.04 $\pm$ 0.29	
	T	1,810 $\pm$ 110	15,430 $\pm$ 460	9.54 $\pm$ 0.63	121 $\pm$ 10%*
7.3%	N	1,020 $\pm$ 50	8,730 $\pm$ 230	9.53 $\pm$ 0.54	
	T	980 $\pm$ 40	9,200 $\pm$ 170	10.41 $\pm$ 0.46	110 $\pm$ 9% ^{NS}
	H	1,000 $\pm$ 70	130 $\pm$ 80	1.12 $\pm$ 0.09	2 $\pm$ 1%***

Blood was obtained from two tolerant mice bearing perfect C57Bl skin grafts for 11 weeks and from two age/sex matched normal mice by the method of Festenstein. Blood was allowed to clot for 1 hr at 4°, then serum taken and spun twice at 800g./15 mins. Aliquots of sera were diluted with appropriate amounts of culture medium, then 200 microl added to 0.50 mls of irradiated CBA or C57Bl spleen cells. 30 mins later 0.60 mls of CBA lymph node cells were added to give a final responder:stimulator ratio of 2:1. 0.20 ml aliquots containing 1.0 million viable responder cells were incubated in the wells of a microtest plate for 71 hrs then labelled with H-3 thymidine for a further 21 hrs. Cultures containing appropriate quantities of hyperimmune serum were included as positive control. Figures for DNA synthesis are cp5m/50 microl. of culture. In the last column the response in the presence of tolerant or hyperimmune serum is shown as a percentage of that obtained in the presence of normal serum. This was calculated as $(I_{T,H}-1)/(I_N-1) \times 100\%$, where I_T , I_H and I_N are the indexes of transformation in the presence of the appropriate serum. The significance of the differences from 100% is shown.

experiment followed closely that of Hellström et al (393). Fresh serum⁺ was added to stimulator (target) cells at concentrations up to 13% and cultures were left at room temperature for about 30 mins. Effector cells were added, approximately halving the serum concentration, and cultures incubated in microtest plates for 3 days before labelling with thymidine. At no concentration tested did tolerant serum cause any detectable inhibition of the responsiveness of normal cells to the tolerising strain of antigen. By contrast, hyperimmune serum gave highly significant inhibition even at the lowest concentration tested. The inability of tolerant serum, even at concentrations as high as 7%, to inhibit the MLC response makes it most unlikely that tolerant serum contains substances able to inhibit the proliferation of cells.

The results of two further experiments are shown in Table 48. In these experiments sera were obtained from individual CBA mice tolerant to A, bearing perfect A strain skin grafts for 2 months (Expt. 2) or 10 months (Expt. 1), except for mouse T4 in Experiment 2 whose graft was showing signs of some rejection. Control sera were from age/sex matched normal CBA mice. Sera were tested for their ability to inhibit the MLC reaction between CBA and (CBA x A)_{F₁} lymph node cells. Stored CBA anti-A hyperimmune serum was added to some cultures as a positive control.

None of the sera obtained from tolerant mice with perfect skin grafts inhibited the MLC responsiveness of normal cells (the stimulations and indexes obtained in the presence of individual tolerant sera in no case differed significantly from the mean values for normal sera). Nor did the mean values of stimulation

+ In this experiment, as in all experiments in this section and section 5, serum was neither heated nor filtered.

TABLE 48. Can serum from tolerant mice inhibit the MLC response of normal cells? II.

Serum concn.	Serum tested	Mean background in unmixd cultures	Stimulation in mixed cultures	Mean Stimulation	Index	Mean Index
<u>Experiment 1.</u>						
6%	N1	2,340 $\pm$ 170	4,260 $\pm$ 710	4,120 $\pm$ 450	2.82 $\pm$ 0.36	2.98 $\pm$ 0.28
	N2	1,860 $\pm$ 100	3,990 $\pm$ 550		3.15 $\pm$ 0.34	
	T1	2,010 $\pm$ 110	3,380 $\pm$ 270 ^{NS}	4,020 $\pm$ 320 ^{NS}	2.68 $\pm$ 0.20 ^{NS}	2.94 $\pm$ 0.19 ^{NS}
	T2	2,140 $\pm$ 200	4,570 $\pm$ 750 ^{NS}		3.14 $\pm$ 0.45 ^{NS}	
	T3	2,140 $\pm$ 120	4,110 $\pm$ 550 ^{NS}		2.92 $\pm$ 0.30 ^{NS}	
	H	1,200 $\pm$ 40	200 $\pm$ 90***		1.23 $\pm$ 0.08***	
<u>Experiment 2.</u>						
3%	N1	3,100 $\pm$ 240	8,220 $\pm$ 1,040	9,820 $\pm$ 560	3.65 $\pm$ 0.43	3.38 $\pm$ 0.20
	N2	5,580 $\pm$ 320	12,360 $\pm$ 880		3.21 $\pm$ 0.24	
	N3	3,860 $\pm$ 300	8,900 $\pm$ 980		3.30 $\pm$ 0.35	
	T1	2,760 $\pm$ 160	8,660 $\pm$ 1,080 ^{NS}	10,280 $\pm$ 410 ^{NS}	4.14 $\pm$ 0.46 ^{NS}	3.89 $\pm$ 0.18 ^{NS}
	T2	4,220 $\pm$ 180	11,240 $\pm$ 480 ^{NS}		3.66 $\pm$ 0.19 ^{NS}	
	T3	3,940 $\pm$ 180	10,960 $\pm$ 360 ^{NS}		3.78 $\pm$ 0.19 ^{NS}	
	T4	3,160 $\pm$ 200	6,060 $\pm$ 400**		2.92 $\pm$ 0.25*	
	H	2,200 $\pm$ 120	2,000 $\pm$ 260***		1.90 $\pm$ 0.15***	

In experiment 1 serum T1 was from a 12-month old tolerant male mouse, and serum N1 from a 14-month old male normal mouse. These sera had been obtained after allowing blood to clot for 3 hrs at 4°, and had been kept in sealed ampoules at -20° for 4 weeks. Sera T2, T3 and N2 were obtained from 12-13 month old tolerant and normal CBA mice, the blood being allowed to clot overnight at 4° and the sera added to culture immediately.

In experiment 2 serum was prepared from the blood of 4 individual 3-month old (but see below) mice and from 3 normal age/sex matched mice. The blood was allowed to clot overnight at 4°, then added to cultures immediately.

All cultures were labelled with H-3 thymidine after 72 hrs culture for a further 16 hrs. All figures for DNA synthesis are cp10m/100 microl. of culture.

TABLE 49. Testing serum from old "tolerant" mice.

Serum concn.	Serum tested	Mean background in unmixed cultures	Stimulation in mixed cultures	Index
<u>Experiment 1.</u>				
5%	N1	1,460 $\pm$ 110	2,670 $\pm$ 420	2.83 $\pm$ 0.34
	N2	1,820 $\pm$ 250	2,590 $\pm$ 510	2.42 $\pm$ 0.41
	T1	1,790 $\pm$ 90	3,100 $\pm$ 310 ^{NS}	2.73 $\pm$ 0.22 ^{NS}
	T2	1,100 $\pm$ 90	1,120 $\pm$ 240**	2.01 $\pm$ 0.26 ^{NS}
	T3	940 $\pm$ 70	1,130 $\pm$ 260**	2.20 $\pm$ 0.32 ^{NS}
	T4	1,320 $\pm$ 120	570 $\pm$ 270***	1.43 $\pm$ 0.23**
<u>Experiment 2.</u>				
3%	N1 + N2	1,930 $\pm$ 140	4,920 $\pm$ 440	3.55 $\pm$ 0.34
	T2 + T4	1,230 $\pm$ 70	1,660 $\pm$ 250***	2.35 $\pm$ 0.23**
	H	1,340 $\pm$ 50	1,750 $\pm$ 150***	2.31 $\pm$ 0.14**
0.3%	N1 + N2	3,330 $\pm$ 170	7,770 $\pm$ 880	3.33 $\pm$ 0.31 ^{NS}
	T2 + T4	2,620 $\pm$ 130	4,900 $\pm$ 940*	2.87 $\pm$ 0.38 ^{NS}
	H	2,630 $\pm$ 230	5,140 $\pm$ 1,070*	2.95 $\pm$ 0.47 ^{NS}

Blood was obtained from four 12-month old CBA mice "tolerant" to A and two age/sex matched normal mice (all mice were first anaesthetised then bled with a syringe by cardiac puncture), and allowed to clot for 6 hrs at 4°. Serum was prepared, stored overnight at 4°, and added to mixed and unmixed cultures of CBA and (CBA x A)F₁ lymph node cells at a concentration of 5% (Expt. 1). The remains of each serum were frozen to -20°. 2½ months later sera were unfrozen, N1 + N2 and T2 + T4 sera pooled, and tested in comparison to hyperimmune serum (H) for ability to inhibit MLC. In both experiments cultures were labelled after 73 hrs for 17 hrs. Figures for INA synthesis are cp10/100 microl. The tolerant donors were grafted with A strain skin at 2 months of age and at the time of testing T1 and T4 had perfect grafts. T2 and T3 had imperfect grafts indicating some degree of rejection.

and index in the presence of tolerant sera differ significantly from the corresponding values for normal sera. Yet in both experiments hyperimmune serum caused strong inhibition of MLC responsiveness. The only tolerant serum to cause any inhibition was that of mouse 24 in Experiment 2. As mentioned above, the graft on this mouse was showing clear signs of rejection at the time of testing, and cannot be considered fully tolerant.

By contrast, tests performed on a group of old tolerant mice showed that serum blocking factors could be detected in a proportion of these mice especially where there was some sign of graft rejection. The results of these experiments are shown in Table 49. Interestingly, the most inhibitory serum was from a mouse with a perfect graft, and when a serum pool formed from the two most inhibitory sera was tested some weeks later, the degree of inhibition was comparable to that given by hyperimmune serum.

A summary of all results obtained with sera from tolerant mice is shown in Table 50.

In each experiment serum was present at a concentration of 3 - 6%, and in some experiments lower concentrations of serum were also tested. In no case did serum from tolerant mice 8-17 weeks old (all having perfect skin grafts) give any detectable inhibition of the MLC response between normal CBA cells and stimulator cells bearing the tolerising strain of antigen (CBA mice tolerant to both A and C57Bl were included in this study). By contrast, in old mice, blocking factors could sometimes be demonstrated, but since their presence was mainly confined to those mice showing some evidence of graft breakdown it would seem that they are only produced after a breakage of tolerance has occurred.

Table 50. Summary of results of attempted MLC inhibition with serum from tolerant mice.

Age of tolerant donors	Sera tested	State of skin grafts	Number of sera causing MLC inhibition
8 weeks	1 pool from 3 mice	Perfect	0/1
11-12 weeks	4 individual sera	Perfect	0/4
16-17 weeks	3 individual sera	Perfect	0/3
	1 pool from 2 mice	Perfect	0/1
12-13 months	5 individual sera	Perfect	1/5
	3 individual sera	Imperfect	3/3

The inability of serum from fully tolerant mice to inhibit the MLC reactivity of normal cells may have come about because any putative blocking factors which it contains act only at the effector stage of the immune response and not at the inductive MLC stage. It seems unlikely that the mechanism causing maintenance of tolerance would allow the wasteful proliferation of cell clones and block only the final effector cells. Nevertheless, the possibility was investigated that serum from tolerant mice might inhibit killing in the chromium release test and the results are shown in Table 51. As can be seen, concentrations of tolerant serum up to 20% caused no detectable inhibition of immune killing. Hyperimmune serum, on the other hand, gave strong inhibition at all concentrations. It can therefore be concluded that serum from tolerant mice contains no substance able to inhibit killing in a Cr-release test.

Table 51. Can serum from fully tolerant mice inhibit killing by in vitro sensitised cells ?

Concn. of serum	Serum tested	Cytotoxicity	Inhibition of cytotoxicity
<u>Experiment 1.</u>			
2%	N	67.3 $\pm$ 0.8%	
	T	66.2 $\pm$ 1.4%	2 $\pm$ 2 ^{NS}
	H	31.4 $\pm$ 0.9%	53 $\pm$ 1%
10%	N	56.0 $\pm$ 1.3%	
	T	58.0 $\pm$ 1.1%	-4 $\pm$ 3 ^{NS}
	H	13.4 $\pm$ 0.8%	76 $\pm$ 2 ^{***}
<u>Experiment 2.</u>			
20%	N	61.8 $\pm$ 1.9%	
	T	60.0 $\pm$ 1.6%	1 $\pm$ 4 ^{NS}
	H	14.2 $\pm$ 1.7%	77 $\pm$ 3 ^{***}

In Experiment 1 blood was obtained from a pool of two 4½-month old tolerant CBA female mice bearing perfect C57Bl grafts for 3 months, and from two normal 3-month old CBA females. It was allowed to clot for 2 hrs at room temperature, then serum prepared. Serum samples were added immediately to aliquots of Cr-51 labelled EL4 cells followed by the addition of CBA spleen cells which had been cultured for 4½ days with either irradiated CBA spleen cells or irradiated C57Bl spleen cells at a 2:1 responder: stimulator ratio. The final effector:target cell ratio was 100:1. Replicates were dispensed to a microtest plate and incubated for 6 hrs.

In Experiment 2, blood was taken from a pool of two 9-week old tolerant CBA mice bearing perfect A skin grafts for 3 weeks from two normal age/sex matched CBA mice. Blood was allowed to clot for 2 hrs at room temperature, serum prepared, and added immediately to Cr-51 labelled A lymphoma cells. CBA lymph node cells sensitised for 4½ days against either irradiated CBA or irradiated A strain spleen cells at a 2:1 responder:stimulator ratio were added to give a final effector:target cell ratio of 125:1. Replicates were incubated in a microtest plate for 6 hrs.

In both experiments appropriate hyperimmune serum raised in CBA strain mice and stored at -80° was added to some cultures as a positive control.

TABLE 52. The MLC reactivity of cells from partially tolerant mice.

Cells tested	Mean background in unmixed cultures	Index of transformation	Reactivity compared to normal cells	
			Individual	Mean
Group A.				
<u>Experiment 1: 4 w old male mice.</u>				
pT1	15,240 $\pm$ 330	0.94 $\pm$ 0.03	-13% NS	-5 $\pm$ 6% NS
pT2	15,800 $\pm$ 420	0.93 $\pm$ 0.04	-14% NS	
pT3	13,780 $\pm$ 280	1.03 $\pm$ 0.05	7% NS	
pT4	14,930 $\pm$ 490	0.99 $\pm$ 0.08	-2% NS	
N	16,200 $\pm$ 90	1.49 $\pm$ 0.05		
<u>Experiment 2: 6 w old male mice.</u>				
pT1	4,730 $\pm$ 320	1.86 $\pm$ 0.13	65%***	24 $\pm$ 3%***
pT2	7,250 $\pm$ 150	1.14 $\pm$ 0.02	11%***	
pT3	5,550 $\pm$ 140	1.29 $\pm$ 0.06	22%***	
pT4	7,450 $\pm$ 180	1.00 $\pm$ 0.06	0% NS	
pT5	7,940 $\pm$ 180	1.00 $\pm$ 0.04	0% NS	
N	7,200 $\pm$ 130	2.33 $\pm$ 0.06		
<u>Experiment 3: 11 w old female mice.</u>				
pT1	6,400 $\pm$ 280	0.96 $\pm$ 0.04	-3% NS	0 $\pm$ 2% NS
pT2	7,000 $\pm$ 180	0.90 $\pm$ 0.04	-6% NS	
pT3	5,140 $\pm$ 220	1.09 $\pm$ 0.06	6% NS	
pT4	5,340 $\pm$ 200	1.12 $\pm$ 0.06	8% NS	
pT5	5,900 $\pm$ 200	0.96 $\pm$ 0.06	-3% NS	
N	5,060 $\pm$ 200	2.51 $\pm$ 0.12		
Group B.				
<u>Experiment 4: 6 w old male mice.</u>				
pT1	14,830 $\pm$ 390	1.28 $\pm$ 0.05	54%***	72 $\pm$ 9%***
pT2	15,350 $\pm$ 400	1.38 $\pm$ 0.05	74%***	
pT3	15,230 $\pm$ 400	1.49 $\pm$ 0.05	96%***	
pT4	15,280 $\pm$ 390	1.33 $\pm$ 0.04	64%***	
N	15,350 $\pm$ 410	1.52 $\pm$ 0.06		
<u>Experiment 5: 6 w old female mice.</u>				
pT1	7,100 $\pm$ 300	1.35 $\pm$ 0.10	28%***	25 $\pm$ 5%***
pT2	7,890 $\pm$ 310	1.33 $\pm$ 0.08	27%***	
pT3	6,820 $\pm$ 320	1.17 $\pm$ 0.10	14% NS	
pT4	7,850 $\pm$ 280	1.40 $\pm$ 0.10	31%***	
N	7,910 $\pm$ 260	2.26 $\pm$ 0.12		

Two groups, A and B, of CBA mice that had been injected ip with $12\frac{1}{2}$ million (CBA x A) F_1 spleen cells at birth were tested. Lymph node cells from these mice (pT) and from pools of three age/sex matched normal CBA mice (N) were cultured in MLC with (CBA x A) F_1 cells. Cultures were labelled after 72-78 hrs with H-3 thymidine for a further 12-20 hrs. All figures for DNA synthesis are cpm/50 microl. of culture. The significance of the responsiveness of cells from partially tolerant mice is shown.

5. MLC studies of partially tolerant mice.

In Section 2 above, the effects of giving lower than a fully tolerogenic dose of (CBA x A) F_1 cells to newborn CBA mice were described. When the size of the cell inoculum was $12\frac{1}{2}$ million only 3/48 mice showed indefinite retention of perfect A strain allografts in adult life. However, 20/48 mice behaved as if partially tolerant, that is, they rejected test allografts only very slowly, sometimes over a period of several months. A similar proportion of mice (4/8) given this dosage of cells at birth were found in a previous study to have serum factors able to inhibit killing by immune cells in the microcytotoxicity test (486). The implication is that mice, for which the dose of F_1 cells administered at birth is just insufficient to cause full tolerance, i.e., the complete elimination or inactivation of potentially reactive clones, mount an ineffectual immune response against the F_1 cells they contain and that this in turn leads to the production of serum blocking factors rather than a destructive immune reaction.

Since the lymph nodes of these mice do not contain cytotoxic cells (486) the question arises whether they are able to recognise the tolerogenic strain of antigen. MLC tests were therefore performed on two separate groups of mice given $12\frac{1}{2}$ million (CBA x A) F_1 cells at birth. The results are shown in Table 52. Those mice tested in MLC were not skin-grafted, but other mice from each group were grafted at 6 weeks of age; the survival of skin grafts on mice in Group A is shown in Table 39, Experiment 3; and of skin grafts on mice of Group B in Table 39, Experiment 4.

The MLC tests showed that 22/22 mice given $12\frac{1}{2}$ million F_1 cells at birth had reduced reactivity compared to normal cells;

Table 53. Attempts to inhibit the reactivity of normal cells with serum from partially tolerant mice.

Serum concn.	Serum tested	Mean background in unmixed or syngeneic cultures	Stimulation in mixed culture	Mean stimulation	Index	Mean index
<u>Experiment 1: Sera from mice of Experiment 1 in Table 52.</u>						
3%	N1	6,300 $\pm$ 310	18,400 $\pm$ 690	19,270 $\pm$ 710	3.92 $\pm$ 0.27	4.48 $\pm$ 0.45
	N2	4,410 $\pm$ 210	19,620 $\pm$ 590		5.45 $\pm$ 0.28	
	N3	6,140 $\pm$ 250	19,790 $\pm$ 1,940		4.22 $\pm$ 0.36	
	T1	6,420 $\pm$ 660	20,120 $\pm$ 1,510	20,040 $\pm$ 810	4.13 $\pm$ 0.47	4.44 $\pm$ 0.47 ^{NS}
	T2	7,330 $\pm$ 280	18,680 $\pm$ 2,170		3.54 $\pm$ 0.32	
	T3	5,780 $\pm$ 180	20,310 $\pm$ 1,100		4.51 $\pm$ 0.25	
	T4	4,300 $\pm$ 370	21,080 $\pm$ 1,400		5.90 $\pm$ 0.60	
	H					
<u>Experiment 2: Sera from mice of Experiment 2 in Table 52.</u>						
5%	N1	2,550 $\pm$ 220	3,470 $\pm$ 470	3,190 $\pm$ 390	2.36 $\pm$ 0.26	2.58 $\pm$ 0.14
	N2	2,020 $\pm$ 160	3,680 $\pm$ 210		2.82 $\pm$ 0.23	
	N3	1,530 $\pm$ 70	2,420 $\pm$ 190		2.58 $\pm$ 0.17	
	T1	2,760 $\pm$ 270	5,350 $\pm$ 400	4,310 $\pm$ 280	2.93 $\pm$ 0.31	2.61 $\pm$ 0.10 ^{NS}
	T2	2,680 $\pm$ 60	4,110 $\pm$ 420		2.53 $\pm$ 0.16	
	T3	2,400 $\pm$ 150	4,110 $\pm$ 200		2.71 $\pm$ 0.18	
	T4	2,700 $\pm$ 140	4,270 $\pm$ 570		2.58 $\pm$ 0.24	
	T5	2,810 $\pm$ 140	3,710 $\pm$ 330		2.32 $\pm$ 0.16	
	H	980 $\pm$ 170	650 $\pm$ 260		1.66 $\pm$ 0.35**	
<u>Experiment 3: Sera from mice of Experiment 3 in Table 52.</u>						
5%	N1	3,000 $\pm$ 180	6,020 $\pm$ 480	6,040 $\pm$ 760	3.01 $\pm$ 0.23	3.16 $\pm$ 0.12
	N2	3,500 $\pm$ 160	7,360 $\pm$ 680		3.10 $\pm$ 0.24	
	N3	1,980 $\pm$ 140	4,740 $\pm$ 280		3.39 $\pm$ 0.27	
	T1	3,160 $\pm$ 220	5,200 $\pm$ 460	5,840 $\pm$ 240	2.64 $\pm$ 0.22	2.82 $\pm$ 0.11*
	T2	3,080 $\pm$ 200	6,220 $\pm$ 700		3.02 $\pm$ 0.29	
	T3	3,140 $\pm$ 220	5,420 $\pm$ 480		2.72 $\pm$ 0.23	
	T4	2,980 $\pm$ 220	6,480 $\pm$ 400		3.17 $\pm$ 0.26	
	T5	3,640 $\pm$ 200	5,880 $\pm$ 500		2.61 $\pm$ 0.19*	
	H	1,040 $\pm$ 80	900 $\pm$ 160		1.86 $\pm$ 0.20**	

Blood was obtained from the mice of Group A whose cellular activities in MLC are shown in Table 52. It was allowed to clot for 5 - 6 hrs at 4°, serum prepared and stored overnight at 4°. Serum was added to mixed lymphocyte cultures between normal CBA lymph node cells and (CBA x A)F₁ spleen cells at a concentration of 3% in Experiment 1, and to cultures of CBA lymph node cells with either irradiated CBA or irradiated A spleen cells at a concentration of 5% in Experiments 2 and 3. Cultures were labelled with H-3 thymidine after 73-75 hrs for a further 16-19 hrs. All figures for DNA synthesis are cpm/100 microl. of culture.

the reduction was usually highly significant and only in 2 cases (mice pT2 and pT3 of Experiment 4 in Group B) was the reduction not significant at the $p = 0.05$ level. Indeed in many cases (12/22) there was no detectable MLC reactivity at all, an occurrence especially common in mice of Group A.

The generally low reactivity of cells from these mice in MLC is in agreement with the prolonged graft survival found in many such mice. However, the agreement between MLC and skin grafting data is not perfect. Thus, while 11/14 mice tested in Group A had no detectable MLC reactivity only 1/13 mice when grafted behaved as fully tolerant. It appears therefore that the MLC tests in vitro are failing to detect small reactivities that must be present; this is to be expected with this strain combination, because the precision of the technique is insufficient to detect reactivities in individual mice of less than 10% of normal.

Attempts to demonstrate factors in the serum of partially tolerant mice capable of inhibiting the MLC reactivity of normal cells have not been reproducibly successful. The results of experiments in which sera from mice of Group A (whose cellular MLC reactivities were shown in Table 52) were tested are shown in Table 53. In 2/3 experiments there was no detectable inhibitory activity. However, in the third experiment the pooled data from the sera of 5 partially tolerant mice indicated a slight inhibition of MLC compared with normal serum, and three of the individual sera showed distinct signs of inhibitory activity.

Blocking activity was more consistently demonstrable with serum from CBA mice which had been given the normally fully tolerogenic dose of $(CBA \times A)F_1$ cells at birth but whose test skin grafts showed signs of chronic breakdown indicating that only partial

tolerance had been achieved. 5/5 such mice had serum which caused inhibition of MLC reactivity, although once again the degree of inhibition was only slight. The results for 3 of these mice are shown in Table 48 (mouse T4) and Table 49 (mice T2 and T3).

6. Discussion.

The results presented in Section 1 of this chapter showed that the ease of tolerance induction in CBA mice was much more closely correlated with the strength of the LD antigens possessed by the donor cells than with the number of serologically defined differences. The LD antigens are already known to play a major role in the induction of GVH (407) and MLC (367-369) reactions and there is evidence to support their having an important role in determining the fate of transplanted tissues (490). The results obtained here suggest that these antigens also have an important role in controlling tolerance induction. The extreme difficulty of obtaining tolerance in the DBA/2 $\rightarrow$ CBA combination hints at the possibility that not only the H2-linked LD loci but also the non-H2-linked (M) LD locus is participating. As only 3 strain combinations have been examined so far, further studies are clearly needed to substantiate the hypothesis presented here. By using congenic H2 recombinant mice there should be no problem in obtaining decisive evidence.

A possible alternative explanation is that Sk antigens are responsible for the difficulty of tolerance induction in the C57B1 $\rightarrow$ CBA and DBA/2 $\rightarrow$ CBA combinations. Thus, many years ago Billingham and Brent reported that when neonatal A strain mice were given (CBA x C57)F₁ cells at birth subsequent grafts of (C57 x CBA)F₁ skin were rejected yet the spleens of these animals were still found to possess lymphoid chimaerism (5). Subsequent

studies by Boyse and colleagues (213,491) have provided strong evidence that C57B1 and A strain mice have different skin-specific alloantigens. Antibodies raised against these antigens do not react with lymphoid cells (492). However, there are good reasons for believing that possible differences in Sk antigens were not responsible for the difficulty of tolerance induction in some strain combinations:

(i) The Sk.2 antigen possessed by C57B1 mice is only very weakly immunogenic: the majority of A strain mice do not reject Sk.2 incompatible grafts within 170 days (213). Yet most CBA mice in which tolerance induction was attempted by injection of large numbers of (CBA x C57B1) F_1 cells at birth subsequently rejected their test skin grafts in less than 30 days.

(ii) In the A $\rightarrow$ CBA combination, where Sk antigen differences, if they exist at all, are extremely weak, 10-12 million F_1 cells given iv at birth was sufficient to induce tolerance in 100% of recipients (Table 36). One CBA mouse given 15-20 million (CBA x C57B1) F_1 cells showed a chronic breakdown of its skin graft over a period of weeks. If this were due to an Sk antigenic difference the mouse should still have been tolerant to its chimaeric lymphoid cells. Yet when tested in MLC the lymphocytes of this mouse were fully reactive to the tolerising strain of lymphocytes (Table 42, mouse T5). Thus the difficulty of tolerance induction in the C57B1 $\rightarrow$ CBA combination must be due to antigenic differences present on lymphoid cells.

(iii) Sk antigen is a true differentiation antigen (492). If it were responsible for the difficulties of tolerance induction in the C57B1 $\rightarrow$ CBA or DBA/2 $\rightarrow$ CBA combinations it should not be possible to overcome the difficulties of tolerance induction by

increasing the dose of lymphoid cells inoculated at birth. Table 36 shows that increasing the numbers of injected F_1 spleen cells increased the incidence of tolerance induction in both these combinations.

(iv) Sk is not linked to H2 (492). Therefore, the ease of inducing tolerance in H2-compatible combinations indicates that Sk differences do not influence tolerance induction.

If the hypothesis that LD antigens control tolerance induction can be verified there may be important implications for the mechanism of tolerance induction, since regions of the major histocompatibility complex which contain strong MLC antigens have only weak ability to induce antibody formation. This partial, or possibly complete, inertness of MLC antigens at inducing antibody formation implies that antibodies or antigen-antibody complexes would not be available to mediate either the induction or maintenance of neonatally-induced tolerance to allogeneic cells.

A rather interesting contrast is found with CBA mice which have been made unresponsive in adult life by treatment with antigen extract and ALS. In these mice unresponsiveness can be induced to A, C57B1, and DBA/2 strains of antigen with approximately equal ease (493), and there is good evidence that the unresponsiveness is caused by active suppression (see Chapter 6). The fact that different antigens seem to be important in the two systems is in line with the apparently different mechanisms involved in maintaining the unresponsiveness.

A further observation along these lines is that tolerance could be induced to M locus antigens, which are also not serologically defined (382). In the CBA v. DBA/2 combination there is almost certainly a strong M locus incompatibility (see footnote to Table 37),

yet lymphocytes from CBA mice made tolerant to DBA/2 showed no MLC responsiveness when cultured with irradiated (CBA x DBA/2) F_1 cells (Table 43). Similarly, cells from CBA mice made tolerant to A showed no MLC reactivity against (CBA x C3H) F_1 cells in vitro (Table 40); C3H and A strain cells are identical at the M locus (488). Actually, in this latter system CBA mice tolerant to A were also partially tolerant to C3H. Although this might suggest that the M locus was playing a role in graft rejection in this combination, evidence from other studies suggests that M locus antigens are unable to influence graft survival (494); the in vivo effect was probably due to A and C3H mice sharing other non H2 tolerogenic determinants.

Some support for the notion that MLC antigens play a role in the induction of self-tolerance comes from the finding that when neonatal mouse thymocytes are mixed with adult syngeneic spleen cells a strong MLC reaction ensues (495-498). This syngeneic reaction is maximal with thymocytes from 2-4 day old mice and over the following 2-3 weeks declines to low levels. The residual low level of syngeneic reactivity present later than 2-3 weeks is probably caused by culture artifacts (see Chapter 3, Section 9a). It seems reasonable to postulate that the observed loss of syngeneic MLC reactivity reflects the induction of T cell tolerance to self MLC antigens and provides support for the concept of clonal elimination/inactivation as a means of avoiding responsiveness to self antigens (455).

The results of MLC tests on lymph node and spleen cells of fully tolerant mice showed no sign of reactive cells. None out of 20 tests on tolerant mice 2-4 months old showed any significant responsiveness in the tolerant cells, and the pooled data from all of these

tests showed no trace of reactivity (Table 44). In 2/7 old tolerant mice there was a hint of some reactivity, and although this may have been the result of an age-dependent breakage of tolerance similar to that which is responsible for the increased incidence of autoimmunity in aging animals (431) a more likely explanation is that the apparent responsiveness was due to the technical difficulties in measuring the low responsiveness of aged mice. Furthermore, in one out of the two cases where cells from old tolerant mice gave a positive response, live (non-irradiated) F_1 stimulator cells were used. Subsequent studies showed that mixtures of cells from old and young syngeneic mice frequently gave levels of DNA synthesis slightly above background.

The total absence of MLC responsiveness in the peripheral lymphoid cells of tolerant mice supports the previous evidence (see Introduction to this Chapter) indicating that neonatally induced tolerance is the result of clonal elimination or inactivation; it is incompatible with the hypothesis of Hellström et al (393) that tolerance is due to an active suppression of reactive cells. It might be argued that the absence of MLC reactivity was caused by either the transfer of blocking factors into the culture system or the continued operation of an active suppressor system in vitro. However, these possibilities are extremely unlikely in view of the following observations:

(i) The reactivity of tolerant cells was not merely greatly reduced, it was totally absent. In the great majority, if not all, other systems in which there is evidence for an active suppression of immunity, at least a trace of reactivity can be detected by appropriate tests.

(ii) Cells washed up to 5 times over a period of 3 hrs before culture were still unable to respond to the specific antigen.

(iii) Attempts to demonstrate factors in the serum of tolerant mice able to inhibit either the MLC reactivity of normal cells or the cytotoxic activity of sensitised cells were completely negative with the exception of some serum samples from very old (12-13 months) tolerant mice which showed distinct signs of inhibitory activity (Table 49). In 3/4 of these latter mice there was some evidence of graft breakdown. Furthermore, no cytotoxic activity was present in lymphoid cells taken from tolerant mice, nor could cytotoxic activity be generated during MLC culture.

(iv) When normal cells were mixed with either an equal number of tolerant cells or with a 3-fold excess of tolerant cells their responsiveness was unimpaired.

How can the findings of Hellström et al (393) be explained in view of the clear absence of MLC reactive cells from the peripheral lymphoid organs of tolerant mice? What seems to be a likely explanation has been revealed from studies of partially tolerant mice. As mentioned in Section 1, the dose of 50 million F_1 cells that Hellström et al used to make A strain mice tolerant to CBA has in the author's hands been quite inadequate, less than one-third of newborn A strain mice so treated showing long-lasting tolerance. Furthermore, although in the reverse direction 50 million F_1 cells has proved an adequate tolerogenic stimulus in CBA mice in this laboratory, at the time when Hellström et al did their experiments the CBA mice at the National Institute for Medical Research, London, were going through a period of immunological hyper-reactivity which may well have caused the normally tolerogenic dose of 50 million F_1 cells to

be inadequate. Although comparisons between mouse colonies in different laboratories are not strictly valid because of differences in environmental factors which may influence the immune system (as suggested in the present studies by the increased difficulty of tolerance induction in the CBA A combination compared to that previously reported - see Section 1 of this chapter) there is certainly a strong possibility that the tolerance-inducing schedules of Hellström et al were insufficient, and, because the mice tested by these workers were not grafted, they had no direct evidence that the particular mice used in their tests were in fact tolerant.

The question therefore arose as to what kind of results would be obtained if mice were deliberately given an insufficiently tolerogenic dose of F_1 cells at birth. The survival of skin grafts on CBA mice given 25, $12\frac{1}{2}$ or 5 million (CBA x A) F_1 cells at birth instead of the normal 50 million have been described in Section 2. Ungrafted mice from the same groups of animals that provided the grafting data for Figure 15 were tested for levels of chimaerism and for the presence of cytotoxic cells and serum blocking factors using a microcytotoxicity test closely similar to that employed by Hellström et al. The latter experiments were performed in collaboration with Professor L. Brent and Drs. Beverley, Simpson, and Medawar of the Clinical Research Centre, London: these results have already been published elsewhere (485). Finally, various groups of CBA mice given a dose of $12\frac{1}{2}$ million F_1 cells at birth were tested for the presence of MLC-reactive cells and serum factors able to block the MLC reactivity of normal cells. In most cases mice were tested when 6-8 weeks old, that is, the animals were the same age as those used in the tests of Hellström et al.

As a result of these studies, three stages in the dose-response curve for tolerance induction could be defined.

1. Mice given 50 million cells at birth were with few exceptions permanently and fully tolerant as judged by the indefinite survival of A strain skin grafts. They had high levels of chimaerism and their cells were totally non-reactive in MLC tests. Furthermore, no cytotoxic cells were detectable in these mice using either a chromium release test or the microcytotoxicity test. Serum from these mice was unable to inhibit either MLC reactions or either of the two cytotoxic reactions. By all criteria used these mice lack cells reactive against antigens of the tolerising strain.

2. Mice given $\frac{1}{4}$ of the fully tolerogenic dose of F_1 cells at birth had significantly prolonged graft survival. Only 3/48 such mice behaved as fully tolerant, but almost half of these mice (20/48) were partially tolerant, that is, they initially had perfect skin grafts and could easily have been mistaken for fully tolerant, but over a period of many weeks or even months chronic rejection of their grafts occurred. (This emphasises the importance of prolonged observation of test skin grafts in order to determine whether mice are indeed fully tolerant). All mice tested from this group had significant chimaerism (4/4) and most had subnormal MLC reactivity (20/22). Although cells from these mice were not cytotoxic in the microcytotoxicity test, in 10/22 cases the cells were reactive in MLC. Most important there was evidence indicating that serum from some of these mice could suppress the MLC reactivity of normal cells and in 4/8 cases factors able to inhibit killing in the microcytotoxicity test were present in the serum. Mice given $\frac{1}{4}$ of the full tolerogenic stimulus are therefore not tolerant but do have suppressed responses to the tolerogenic strain of antigen. When

once established this state of active suppression is long-lasting as shown by the extremely slow breakdown of grafts in these mice and the fact that one group of mice when grafted at 6 months of age contained a similar proportion of partially tolerant mice as groups grafted at 6 weeks of age (see Expt. 2, Table 39).

3. Mice given $1/10$ th of the fully tolerogenic dose showed no tolerance to skin grafts and only very slight, if any, lymphocyte chimaerism. Such mice often had cytotoxic cells in the microcyto-toxicity test (4/12) but no serum blocking factors (0/12).

These investigations, coupled with the evidence above that the mice used by Hellström et al were not given sufficiently high numbers of cells at birth to induce complete tolerance, and the fact that they did not test their mice with skin grafts prior to testing in vitro, strongly suggest that these authors were in fact studying a state of partial tolerance. Subsequent work by Hellström and colleagues (499,500) has claimed that rats made tolerant at birth by iv injection of 40 million parental strain bone-marrow cells possess both cytotoxic cells and blocking factors. However, their tolerance induction procedure causes only 40% of "tolerant" rats to retain skin grafts for greater than 100 days. (500). This is little better than the approximately 20% survival of A strain grafts placed on CBA mice given $\frac{1}{4}$ of the fully tolerogenic dose of cells at birth. Furthermore, their use of parental strain cells to induce tolerance provides further problems in the interpretation of their results. Although the inoculation of fully allogeneic bone-marrow cells does not usually cause overt signs of runting, a chronic low grade GVH reaction might be occurring. It has recently been demonstrated that chronic graft versus disease in

mice leads to autoimmunity (501). In view of the inadequacy of their tolerance-inducing schedule and the distinct possibility of chronic graft-versus-host reaction it is not surprising that they have been able to detect cellular and humoral immunity in their animals. It must also be pointed out that serum from mice syngeneic with the target cells has been found to inhibit killing in the microcytotoxicity test (502), suggesting that soluble histocompatibility antigens present in serum can block effector cells. Since tolerant mice are known to be chimaeric there is a possibility that some of the blocking activity claimed to be present in tolerant serum is free antigen.

Voisin and colleagues have also claimed that CBA mice made neonatally tolerant by iv injection of (CBA x A) F_1 cells have serum blocking (facilitating) factors (503). Their mice appear to be genuinely fully tolerant, but their claim that such mice contain synergic antibodies has not been verified in this laboratory, all sera tested (from either young or old tolerant mice) having proved completely negative for either direct or synergic haemagglutinating antibodies (504).

There have also been claims that in tetraparental mice mutually cytotoxic cells and serum blocking factors can be demonstrated in the microcytotoxicity test (505). Furthermore, Philips et al presented evidence that serum from tetraparental mice would inhibit stimulation by parental strain lymphocytes in MLC (506). Philips and Wegmann went on to show that lymphocytes from tetraparental mice would apparently suppress the mixed lymphocyte reactivity of normal cells (507). However, studies by other groups of workers do not support these results. Thus, Ceppellini reported that studies of a tetragametic human chimaera had shown

the lymphocytes to be specifically unreactive in MLC even when either one of the chimaeric subpopulations had been eliminated by pretreatment with anti-HL-A sera (508). Serum from the tetragametic individual was unable to block the reactivity of his own or his siblings' lymphocytes. Similar conclusions have been drawn by Meo et al (509) from a study of tetraparental mice. Tetraparental cells were unreactive to either of the parental types in MLC and serum caused no inhibition of the ability of parental or tetraparental cells to stimulate or to respond to genetically unrelated donors. Furthermore, regardless of whether blocking activity and/or reactive lymphocytes are present in tetraparental individuals, there are serious objections to the use of these animals as models for self-tolerance since the chimaeric state of these mice is not uniform, a number of organs having been found to be composed entirely of cells of a single parental type (510).

Indeed, the evidence supporting clonal elimination/ irreversible inactivation as the mechanism behind neonatally-induced tolerance has continued to mount in recent years. In an extensive investigation Brent et al, using various in vivo assays, found no evidence to support the existence of serum blocking factors in tolerant mice (511). Furthermore, it has been shown that tolerance can be induced in agammaglobulinaemic bursectomised chickens (512), clearly demonstrating that humoral antibody is not required for tolerance induction. The failure of cells from tolerant mice (511) or from tolerant rats (513) to react in the GVH assay has been confirmed. In the latter experiments incubation of normal cells with serum from tolerant rats or mixing them with a 10-fold excess of cells from tolerant rats caused no detectable diminution of their ability to react in the popliteal lymph node GVH assay. Finally, Elkins studied the GVH

reactivity of cells from neonatally tolerant rats in which tolerance had been broken by the transfer of normal cells (514). Although 90% of such rats rejected their skin grafts in 15-25 days, the GVH reactivity of their peripheral lymphocytes in the first month after cell transfer was due almost entirely to the normal cells used to break tolerance (as judged by chromosomal analysis of metaphases), very few of the tolerant animals own lymphocytes participating. After about 4 weeks the participation of cells of host type in the GVH reaction was clearly demonstrable, but if the tolerant rats were thymectomised prior to breakage of tolerance very few of their cells were ever observed to participate in GVH reactions even when tested many months after breakage of tolerance. These remarkable experiments provide strong evidence that the state of transplantation tolerance is not due to reversible inactivation of potentially reactive cells.

Space does not permit any extensive discussion of the interesting observation of Micklem concerning the formation of syn-clusters (515) or of the elegant work of Cohen and colleagues (272, 516,517) suggesting that auto-reactivity can be induced against syngeneic embryonic fibroblasts. The former system lacks clear evidence of specificity and although it is conceivable that auto-reactive cells with very low antigen affinity, such that they cannot be triggered after contact with antigen, may escape clonal elimination it is hard to envisage what advantage such low-affinity-escape could confer on the animal. Cohen et al's experiments are not at all easy to explain other than by the existence of auto-reactive cells against fibroblast antigens. It may well be that the mechanism of clonal elimination/inactivation is reserved for tolerance to the major histocompatibility antigens of the species where the complete

removal of all auto-reactive cells is essential for the survival of the animal. The active suppression against alloantigens which is detectable in cases of partial tolerance (see above) may represent a second line of defence against the possibility of cells escaping the censoring mechanism. By contrast, for the less immunogenic antigens, such as differentiation antigens, active suppression may be the primary defence against autoimmunity and this may explain the ease with which autoimmunity against minor self-antigens can be induced experimentally, and the age-dependent onset of autoimmune symptoms as the power of the immune system, in particular of the suppressor mechanisms, declines. Certainly, if one accepts the existence of Sk antigens as true differentiation antigens, the ability to induce tolerance to skin grafts by neonatal inoculation of lymphocytes strongly suggests that methods other than clonal elimination are available for suppressing immunity to differentiation antigens.

CHAPTER 6.In vitro studies of mice made unresponsive to skin grafts in adult life.

In contrast to the situation in neonatally tolerant mice, previous studies in this laboratory had shown that spleen cells from mice made unresponsive in adult life to allogeneic skin grafts were reactive in the splenomegaly GVH assay (518). In this chapter the results of subsequent in vitro investigations of the immune status of these animals will be described. Because the conclusions drawn at various stages of the investigation were closely inter-related with other studies being performed concurrently in this department, a discussion of the results obtained will be included at appropriate places in the text.

Three month old male CBA mice were made specifically unresponsive to allogeneic skin grafts as described in "Materials and Methods". Only mice which had borne perfect grafts for more than two months were accepted for in vitro tests. The reactivity of cells from unresponsive mice was compared to that of either normal mice or mice treated at the same time as the unresponsive test animals with B. pertussis vaccine and ALS: these latter are referred to as control animals below. The normal and control mice were in all cases of the same age and sex as the unresponsive mice. Similarly, where the effects of serum from unresponsive mice on in vitro responses were tested they were compared to those of serum from age/sex matched normal or control mice bled aseptically at the same time as the unresponsive animals. Blood was obtained in all cases by the method of Festenstein (see Materials and Methods) and, except where otherwise stated, serum was added to cultures immediately after preparation. In no case was serum either heated or filtered.

TABLE 54. MLC tests on the lymph node cells of CBA mice made unresponsive to A strain skin grafts.

Expt.	Responder cells	Mean Background of unmixed cultures	Stimulation by (CBA x A) F_1 cells	Index to (CBA x A) F_1	Index to (CBA x DBA/2) F_1
1	Normal	7,260 $\pm$ 240	3,220 $\pm$ 1,160	1.44 $\pm$ 0.15**	13.9 $\pm$ 0.8
	"Unresponsive" 1	8,260 $\pm$ 460	10,960 $\pm$ 1,180	2.32 $\pm$ 0.17***	12.1 $\pm$ 0.9
	"Unresponsive" 2	6,600 $\pm$ 380	7,120 $\pm$ 1,720	2.08 $\pm$ 0.25***	15.9 $\pm$ 1.5
2	Normal 1	6,570 $\pm$ 530	4,260 $\pm$ 990	1.65 $\pm$ 0.18***	9.4 $\pm$ 1.3
	Normal 2	4,660 $\pm$ 360	2,700 $\pm$ 740	1.60 $\pm$ 0.20***	5.8 $\pm$ 1.2
	"Unresponsive" 1	15,100 $\pm$ 680	7,700 $\pm$ 1,500	1.51 $\pm$ 0.11***	4.7 $\pm$ 0.4
	"Unresponsive" 2	6,330 $\pm$ 390	1,680 $\pm$ 840	1.23 $\pm$ 0.14**	6.2 $\pm$ 0.7

Lymph node cells from unresponsive and age/sex-matched normal mice were cultured with (CBA x A) F_1 and (CBA x DBA/2) F_1 stimulator cells and in control cultures with syngeneic cells. After 3 days cells were labelled with H-3 Tdr for 20 hrs. All figures for DNA synthesis are cp10m/100 microl. of culture. The significance of the response to A strain antigens is shown (indexes compared to 1.00 on 1-tail t-test.) Unresponsive mice had carried perfect skin grafts for 8 months (expt. 1) or 5 months (expt. 2).

1. MLC reactivity of cells from unresponsive mice.

Many of the unresponsive mice tested were considerably older than the F_1 hybrid mice which were available as donors of stimulator cells for MLC. Therefore, to guard against any possibility of "syngeneic effects" between cells of disparate age or sex, an extra control was included in those MLC experiments where non-irradiated F_1 stimulator cells were used. This was to culture cells from unresponsive mice (and also from normal and control mice) with syngeneic cells from mice of the same age and sex as the F_1 cell donors. The slight response usually observed in such cultures was subtracted from that observed in allogeneic mixed cultures to give the nett response to allogeneic antigens.

Table 54 shows the results of two experiments in which lymph node cells from four individual unresponsive mice were tested in MLC. Although all four mice had borne perfect A strain skin grafts for many months, their lymphocytes gave highly significant responses to A strain antigens in vitro. There was a considerable range of reactivity, cells from two of the mice behaving as hyper-reactive, while those from one mouse behaved as hypo-reactive.

To examine more closely the level of MLC responsiveness in the cells of "unresponsive" mice the experiments in Table 55 were performed. In these tests, cells were obtained from small pools of relatively young CBA mice made unresponsive to C57B1 or DBA/2 strain skin grafts. The strong MLC reactivity of CBA cells to these two strains (compared with MLC responses to A strain antigens) allows a relatively precise determination of the level of responsiveness. Furthermore, in most of these experiments the reactivities were compared with those of control rather than normal cells.

Table 55. Level of MLC reactivity in cells from unresponsive mice.

Expt	Responder cells	No. of mice in pool	Mean background in control cultures	MLC response		% response relative to control cells
				Stimulation	Index	
1a	Control LN	2	19,000 $\pm$ 750	57,900 $\pm$ 1,800	4.04 $\pm$ 0.18	60***
	Unresponsive LN	2	31,500 $\pm$ 900	57,500 $\pm$ 2,500	2.82 $\pm$ 0.11	
1b	Control Sp	2	16,900 $\pm$ 700	21,100 $\pm$ 1,400	2.25 $\pm$ 0.12	75*
	Unresponsive Sp	2	16,600 $\pm$ 300	15,700 $\pm$ 1,100	1.94 $\pm$ 0.07	
2	Normal LN	1	6,200 $\pm$ 80	11,450 $\pm$ 750	2.84 $\pm$ 0.13	77 ^{NS}
	Unresponsive LN	1	7,300 $\pm$ 330	10,350 $\pm$ 1,300	2.42 $\pm$ 0.20	
3	Normal LN	3	5,340 $\pm$ 220	31,600 $\pm$ 700	6.92 $\pm$ 0.32	61***
	Unresponsive LN	3	6,860 $\pm$ 280	24,700 $\pm$ 1,000	4.61 $\pm$ 0.26	
4	Control LN	2	3,880 $\pm$ 140	5,800 $\pm$ 270	2.44 $\pm$ 0.11	87 ^{NS}
	Unresponsive LN	3	4,830 $\pm$ 260	6,060 $\pm$ 370	2.25 $\pm$ 0.13	
5	Control LN	2	22,850 $\pm$ 950	30,700 $\pm$ 950	2.34 $\pm$ 0.10	106 ^{NS}
	Unresponsive LN	3	21,300 $\pm$ 400	30,250 $\pm$ 1,050	2.42 $\pm$ 0.06	

The unresponsive mice used for experiments 1 - 4 had been made unresponsive to C57B1 strain antigens and had borne perfect C57B1 skin grafts for 2 months (expts. 1, 3, and 4) or 9 months (expt. 2); those used for experiment 5 had been made unresponsive to DBA/2 antigens and had retained perfect skin grafts for 3 months. Lymphocytes from these animals were cultured in MLC with the appropriate strain of live F_1 cells or (in expts. 4 and 5) with irradiated parental cells. DNA synthesis was measured with a 15-22 hr pulse of H-3 thymidine after either 72 (expts. 2 and 3) or 96 hrs (expts. 1, 4, and 5) of culture. All figures for DNA synthesis are cp5m/100 microl. of culture. In the last column the significance of the differences between normal and "unresponsive" MLC reactivities is shown. The relative response was calculated from the formula $(I_U - 1)/(I_{C,N} - 1)$ where I_U , I_C , and I_N are the indexes of transformation of unresponsive, control, and normal cells respectively.

In 5/6 experiments the MLC responsiveness of cells from unresponsive mice was lower than that of control or normal cells, in 3 cases the depressed response being significant at at least the $p=0.05$ level. The combined results for all six experiments showed that, on average, cells from unresponsive mice had indexes of transformation to the specific strain of antigen 24% lower than did cells from control mice. The difference in reactivity between the two types of cells was highly significant statistically. However, the relevance of this depressed MLC responsiveness to the in vivo situation is more difficult to evaluate for two reasons.

Firstly, although kinetic studies of MLC responses in this culture system clearly demonstrated linear responses on log v log plots (Chapter 3, Section 10), only with irradiated stimulator cells were the responses linear on double arithmetic plots. Therefore, until comparative dose-response experiments can be done with control and "unresponsive" cells it will not be possible to evaluate quantitatively the degree of depression of responsiveness. A second limitation to the experiments described here is that the depressed response has not been tested for specificity. Although age/sex-matched mice treated with pertussis and ALS at the same time as the test animals were used as controls, it would be preferable to use age/sex-matched mice made unresponsive to a third-party strain of skin graft. Both types of mice could then be tested against the specific and third-party cells in MLC. This approach could not, however, be used to quantitate the degree of in vitro responsiveness because of the extensive cross-reactivity between the LD antigens in different strains of mice (see Chapter 5, Section 3e).

If the depressed reactivity of "unresponsive" cells in MLC

is truly specific, it may result from the partial activity of an active suppressor system in vitro. On the other hand, it is possible that the pretreatment with Ag extract and skin grafting, followed by ALS administration, leads to a specific depletion of the antigen reactive clones in which subsequent recovery never occurred to the levels found in normal or control mice. Since unresponsive mice clearly have at least moderate levels of MLC-reactive cells, clonal depletion is an insufficient explanation for the long term unresponsive state, in contrast to the situation in neonatally tolerant mice (Chapter 5). Alternative explanations of the defective MLC responsiveness are that some kind of inhibition is acting on the T cells from these mice. Two possibilities spring to mind. One is that antigen-antibody complexes are initially blocking the surface receptors of antigen-reactive cells. In the absence of further generation of blocking complexes in vitro, the cell bound inhibitors would most likely be released from the cell surface either by dissociation from the receptor molecules or following an active capping or shedding process (519, 520). The de-repressed cells would then enter into the MLC reaction cycle but at a somewhat later stage than did control cells, so that the degree of reaction of these cells would lag behind their control counterparts. A prediction of this model is that the earlier the assay time in MLC the greater would be the difference between the responses of control and "unresponsive" cells. Using the recently developed protein synthesis assays of MLC reactivity (400, 521) it should be possible to measure the MLC responsiveness within about 24 hrs of culture, and thereby acquire information as to whether a de-repression model is tenable.

A second explanation is that suppressor cells are active in

the in vitro system. One would have to postulate that they are less effective in vitro than in vivo, but as there are numerous reasons why this might occur, their participation in the partial suppression of initiator function in vitro must be taken as a real possibility.

The finding that the cells from unresponsive mice are reactive in MLC agrees with earlier studies on the GVH reactivity of cells from mice made unresponsive without the aid of B pertussis vaccine (518). In these studies, spleen cells from the unresponsive mice caused a degree of splenomegaly in newborn F_1 hybrid mice comparable to that induced by injection of normal cells. More recent investigations have shown that mice made unresponsive with the aid of B. pertussis have lymphoid cells which are active not only in the splenomegaly GVH assay but also in the popliteal lymph node GVH assay (522). Genetic analysis of the splenomegaly GVH assay (407) and the MLC reaction (367-369) suggests that these two reactions are controlled by the same antigen, both systems measuring the ability of cells to recognise LD determinants. There is some evidence that the popliteal lymph node assay may be able to detect antigenic differences which cannot cause a splenomegaly GVH reaction (523), but since these extra antigens are probably also of LD type (383) the difference between the two GVH systems may be only one of sensitivity.

2. Generation of cytotoxic cells.

Table 56 shows the results of experiments where attempts were made to generate cytotoxic cells either directly in unresponsive mice or in their lymphoid cells cultured in vitro.

Experiment 1 in this table shows that lymph node cells from unresponsive mice can be readily sensitised in MLC to the antigens

Table 56. Generation of cytotoxic cells following in vitro or in vivo stimulation of cells from unresponsive mice.

Expt.	Mode of stimulation	Effector cells	Stimulator spleen cells	% killing of EL 4 (C57B1) cells	% killing of P815 (DBA/2) cells
1	in vitro	None	-	1.7 ± 0.3	1.3 ± 0.8
		Control	(CBAx C57B1) F_1	57.0 ± 3.0	14.0 ± 2.0
		"Unresponsive"	(CBAx C57B1) F_1	56.0 ± 3.0	13.0 ± 2.0
		"Unresponsive"	(CBAx DBA/2) F_1	6.0 ± 1.0	83.0 ± 8.0
2	in vivo	None	-	-0.4 ± 0.6	N.D.
		Control	(CBAx C57B1) F_1	29.0 ± 1.0	N.D.
		"Unresponsive"	(CBAx C57B1) F_1	11.0 ± 1.0	N.D.
		Control	(CBAx DBA/2) F_1	N.D.	45.0 ± 2.0
		"Unresponsive"	(CBAx DBA/2) F_1	N.D.	46.0 ± 1.0
3	in vivo	None	-	0.8 ± 1.0	N.D.
		"Unresponsive" 1	(CBAx C57B1) F_1	39.0 ± 1.0	N.D.
		"Unresponsive" 2	(CBAx C57B1) F_1	42.0 ± 2.0	N.D.
		"Unresponsive" 3	(CBAx C57B1) F_1	52.0 ± 2.0	N.D.
		Control	(CBAx C57B1) F_1	78.0 ± 1.0	N.D.

In experiment 1, lymph node cells from two unresponsive mice bearing perfect C57B1 skin grafts for two months were cultured either alone or at a 3:1 responder:stimulator cell ratio with either (CBA x C57B1) F_1 or (CBA x DBA/2) F_1 spleen cells. Lymph node cells from two control mice were also cultured either alone or with (CBA x C57B1) F_1 spleen cells. After $4\frac{1}{2}$ days of MLC, cells were harvested and recultured with Cr-51 labelled EL4 or P815 cells at an effector:target cell ratio of 150:1 for $5\frac{1}{2}$ hrs.

In experiment 2, individual unresponsive and control mice (the former having had perfect C57B1 skin grafts for 2 months) were injected ip with 15 million (CBA x C57B1) F_1 or (CBA x DBA/2) F_1 spleen cells, or saline. Five days later spleen cell suspensions were prepared, washing the cells once. They were then incubated with Cr-labelled EL4 cells at a 200:1 effector:target cell ratio for 12 hrs.

Experiment 3 was performed in an identical manner except that the immunisation dose was 24 million cells and recovered spleen cells were washed three times before cytotoxic assay.

of the strain to which they are specifically unresponsive in vivo. Within the limits of cross-reactivity between target cell antigens this in vitro sensitisation was clearly specific and, in this particular experiment the generation of effector cells was equally efficient in "unresponsive" and control lymphocyte populations. Although in 1/2 other experiments (see Table 60) the generation of effector cells occurred less efficiently in cell populations from unresponsive mice than from control mice, their presence could always be demonstrated unequivocally, thus showing that there is no lesion in the pathway of effector cell production in unresponsive mice.

There can therefore be no doubt that an active form of suppression must exist in such mice, the alternative possibility of loss of antigenicity in the test skin graft of such mice having been excluded by the demonstration that (i) injection of pre-immunised cells led to rejection of the test graft (518): (ii) transfer of the test grafts to normal CBA mice resulted in their rejection at approximately normal rate (524), (iii) new grafts placed on long term unresponsive mice show prolonged and sometimes indefinite survival (525).

Further evidence supporting the notion of an active suppression of immunity in these animals has come from the following findings:

- (a) Administration of cyclophosphamide to mice, beginning 15 days after grafting, prevents the establishment of the unresponsive state (526). Administration of ALS over the same time period does not interfere with the induction of unresponsiveness. Interestingly, Turk et al (527) have found that cyclophosphamide elevates a contact sensitivity reaction in guinea pigs. The fact that

cyclophosphamide seems to deplete B-cell areas of the lymphoid tissues preferentially (528) might suggest that the suppressor mechanism in these mice is associated with B cell function. However, the detailed effects of cyclophosphamide are not known and certainly it would be unwise to assume that it does not also interfere with T cell function. Nor does the failure of ALS to interfere with the induction of unresponsiveness indicate the nature of the suppressor cells, for any possible damage ALS inflicts on the suppressor cells would be masked by the non-specific depression of allograft immunity caused by ALS. Surprisingly, administration of cyclophosphamide to long-term unresponsive mice is less effective in breaking the unresponsive state (524), suggesting that in these latter animals there must be a slow turnover of the putative suppressor cells.

- (b) Spleen cells from unresponsive mice transfer unresponsiveness to ALS-treated recipients (526).

The question therefore arises as to the nature of the suppressor mechanism.

The observation that not all second grafts show indefinite survival on unresponsive mice suggests that if these mice are suddenly confronted with a large increase in the amount of antigen the suppressor mechanism can be overstepped. To test this possibility further, long-term unresponsive mice were immunised with a relatively large dose (15 or 24 million) of F_1 spleen cells ip. Five days later spleens were removed from such mice and tested in vitro for the presence of cytotoxic cells. The results are

shown in experiments 2 and 3 of Table 56. In both cases un-immunised control mice had no detectable cytotoxic cells in their spleens. However, after immunisation such cells could be clearly demonstrated in the Cr-release test. The killing activity of spleen cells from immunised unresponsive mice was considerably less than that of spleen cells from immunised control mice against the specific antigen. By contrast, unresponsive mice immunised with third-party cells had normal levels of effector cells against these cells (expt. 2). Thus, just as cells from unresponsive mice show a slightly subdued response in both the proliferative and effector stages of MLC, so also does in vivo immunisation demonstrate a reduced level of responsiveness. Once again, exact quantitation of the degree of suppression would require dose-response studies, and although specificity of the in vivo immunisation was not formally demonstrated numerous workers using very similar systems have shown that the effector cells are specifically sensitised T cells (529, 530).

Although these experiments show that large quantities of antigen can break through the suppressive system operating in these mice, the effector cells generated seem to have little ability to cause graft rejection. Thus repeated ip injection of A strain cells caused breakdown of grafts in only 1/3 mice (524). (Interestingly, the two immunised mice whose grafts remained intact also did not produce haemagglutinating antibodies. Since the production of IgM antibodies against histocompatibility antigens is thymus-independent (531) this finding suggests that not only the T cells but also the B cells of unresponsive mice are subjected to the suppressive effects).

Data such as this are most easily interpreted in terms of a block acting at the efferent level of immunity. However, other results obtained in this laboratory are inconsistent with this idea.

- (a) Despite the fact that a state of presensitisation can be demonstrated with the MLC technique employed in these studies (Chapter 4, Section 4), lymphoid cells from unresponsive mice behave as hyporeactive rather than hyper-reactive in this assay.
- (b) Presensitised cells (but not normal cells) are able to break unresponsiveness in these mice (518). (The overloading of an efferent suppressor system by a sudden infusion of large numbers of sensitised cells cannot, however, be excluded).
- (c) Host versus graft (HVG) assays in these mice give negative results. Thus injection of F_1 cells into the hind footpad of unresponsive mice causes no enlargement of the popliteal lymph node, whereas control mice so treated have considerably enlarged lymph nodes (522). The similarity of this reaction to the popliteal lymph node GVH assay, which is usually considered as an assay for initiator cell function, suggests that it also is a measure of the initial proliferative responses to antigen. Thus the absence of HVG reactions in unresponsive mice suggests a blockage at the afferent or central level of the immune response.
- (d) The failure to detect cytotoxic cells in unresponsive mice prior to immunisation, and the subdued production of effector cells which occurs after immunisation strongly suggest that the suppression acts largely at a point

prior to the production of effector cells.

The only results which at first seemed inconsistent with suppression at the initiation level came from homing studies in which Cr-labelled A strain lymphocytes showed a homing pattern in CBA mice unresponsive to A strain skin grafts which was similar to the pattern found in sensitised mice (i.e. high localisation in livers) rather than that found in normal mice (532). However, studies by R.R.C. New in this department have shown that pre-incubation of A strain lymphocytes with either hyperimmune serum or unresponsive serum causes them to home preferentially to the liver even in normal CBA mice, strongly suggesting that pre-formed antibody is the mediator of altered homing patterns in sensitised and unresponsive mice. Although haemagglutinating antibodies are not normally detectable in the unresponsive mice, recent studies using a modified Coombs test have revealed that these mice do possess small quantities of IgG₁ antibody (526). The presence of this antibody provides an explanation of the observed homing pattern of donor strain lymphocytes.

It therefore seems that the block acting in these unresponsive mice is at the initiation or recognition level.

Because the suppressor mechanism is maintained by the relatively low levels of antigen released from the long established skin graft, it is not difficult to accept that the sudden presentation of a large quantity of antigen would break through the barrier and cause the generation of at least some cytotoxic cells. What is puzzling, however, is that when the antigen is given in the form of F₁ cells the cytotoxic cells that are generated do not always lead to graft breakdown. Similarly, when the antigen

Table 57. Attempts to inhibit the MLC reactivity of normal CBA lymph node cells to (CBA x C57Bl)F₁ stimulator cells with varying concentrations of serum from mice unresponsive to C57Bl skin allografts.

Expt	Serum	Concn. (%)	Mean background of unmixed cultures	Stimulation by (CBA x C57Bl)F ₁ cells	Index
1	Control	0.75	16,500 ± 440	51,300 ± 1,800	4.1 ± 0.2
	"Unresponsive"	0.75	15,300 ± 520	47,800 ± 830	4.1 ± 0.1
	Hyperimmune	0.75	14,200 ± 740	21,800 ± 660	2.7 ± 0.2
	Control	1.5	10,100 ± 490	45,700 ± 3,200	5.5 ± 0.4
	"Unresponsive"	1.5	11,400 ± 500	47,300 ± 2,300	5.2 ± 0.3
	Control	3.0	5,670 ± 390	32,800 ± 2,100	6.8 ± 0.6
	"Unresponsive"	3.0	4,970 ± 220	30,400 ± 1,800	7.1 ± 0.5
	Hyperimmune	3.0	3,770 ± 180	9,300 ± 940	3.8 ± 0.3
	Control	6.0	1,500 ± 100	16,800 ± 1,200	11.3 ± 1.1
	"Unresponsive"	6.0	1,630 ± 180	13,700 ± 2,000	9.4 ± 1.7
2	Normal	0.3	154 ± 22	3,350 ± 500	22.8 ± 4.6
	"Unresponsive"	0.3	137 ± 12	2,720 ± 520	20.9 ± 4.8
	Normal	3.0	108 ± 14	1,460 ± 240	14.5 ± 2.8
	"Unresponsive"	3.0	95 ± 10	1,600 ± 170	17.9 ± 2.6
3	Normal	0.3	2,700 ± 180	10,400 ± 800	4.8 ± 0.4
	"Unresponsive"	0.3	2,170 ± 120	11,500 ± 600	6.3 ± 0.4
	Normal	3.0	1,740 ± 150	8,150 ± 900	5.7 ± 0.6
	"Unresponsive"	3.0	1,460 ± 110	7,550 ± 850	6.2 ± 0.7

In experiment 1 serum was obtained from pools of 2 unresponsive and 2 control mice and stored overnight at 4° before use. For details of hyperimmune serum see Materials and Methods. The unresponsive mice had borne perfect skin grafts for 2 months. Sera for experiments 2 and 3 were freshly obtained from individual mice and were added to cell suspensions at the two dilutions shown. The unresponsive mice retained perfect skin grafts for 6½ months (expt. 2) and 9 months (expt. 3). Cultures were labelled after 97 hrs (expt. 1), 94 hrs (expt. 2), and 72 hrs (expt. 3) for a further 16 hrs. Figures for DNA synthesis are cp5m/100 microl. of culture.

is given in the form of a second skin graft, the primary test graft is in many cases not rejected (525). These findings imply a complex form of initiator cell suppression which may be able to act also on effector cells in certain circumstances.

3. Attempts to demonstrate serum blocking factors.

In a number of systems in which cellular immunity is specifically suppressed, factors able to inhibit cellular responses in vitro have been detected. In some such cases there is evidence that the factors are antigen-antibody complexes, this having been most elegantly demonstrated by Baldwin et al in a hepatoma tumour system in rats (489).

Therefore a detailed study of serum from unresponsive mice was undertaken to examine whether it could inhibit MLC, or Cr-release cytotoxicity assays in vitro.

Table 57 shows the results of the attempts to inhibit the MLC reactivity of normal cells with serum from unresponsive mice. The serum was tested over a 20-fold range of concentrations. In no case did serum from unresponsive mice cause any detectable inhibition of the MLC response. By contrast, hyperimmune serum even at the lowest concentration tested caused a highly significant ($p < 0.001$) depression of MLC reactivity.

It is conceivable that serum factors would be able to maintain a blockade of cells that are already blocked yet be unable to induce a blockage of normal cells. As discussed above, one explanation of the subnormal MLC reactivity of lymphoid cells from unresponsive mice is that they are initially blocked but that subsequent shedding of the blocking material allows them to become reactive in vitro. If such cell-bound blocking substances were derived from the

serum then addition of "unresponsive" serum to cultures of "unresponsive cells might well be expected to potentiate the hyporeactivity of the cells. However, as shown in the experiment of Table 58, although the "unresponsive" cells were indeed less reactive than their normal counterparts, there was no evidence that their subnormal reactivity could be decreased further by the addition of unresponsive serum at a concentration of 6%. Yet hyperimmune serum inhibited very effectively ($p < 0.001$).

Table 58. Attempt to potentiate the subnormal reactivity of cells from unresponsive mice with unresponsive serum.

Responder cells	Serum	Mean background of unmixed cultures	Stimulation by (CBA x C57Bl) F_1 cells	Index
Normal	Normal	580 $\pm$ 80	5,220 $\pm$ 300	10.0 $\pm$ 1.5
	"Unresponsive"	870 $\pm$ 40	7,530 $\pm$ 310	9.7 $\pm$ 0.7
	Hyperimmune	410 $\pm$ 50	820 $\pm$ 80	3.0 $\pm$ 0.4
"Unresponsive"	Normal	890 $\pm$ 60	6,710 $\pm$ 180	7.5 $\pm$ 0.5
	"Unresponsive"	1,360 $\pm$ 50	9,400 $\pm$ 400	7.9 $\pm$ 0.4
	Hyperimmune	910 $\pm$ 100	1,040 $\pm$ 160	2.1 $\pm$ 0.3

Blood and lymph nodes were removed from a pool of 3 CBA mice unresponsive to C57Bl skin grafts (in perfect condition for 7 weeks) and from a pool of 2 normal mice. Serum and cell suspensions were prepared and mixed cultures set up with (CBA x C57Bl) F_1 lymph node stimulator cells. Normal "unresponsive" and hyperimmune sera were added to a concentration of 6%. Cultures were labelled after 72 hrs with H-3 thymidine for 15 hrs. Figures for DNA synthesis are cp5m/100 microl. of culture.

Attempts to inhibit mouse MLC responses with serum are restricted by the high toxicity of normal mouse serum (Chapter 4, Section 2), the maximum concentration that can be used being about 6%. Serum blocking factors, if they exist, might reasonably be expected to show some sign of their presence at such concentrations as judged by the ability of immune (Chapter 4, Section 4) and hyperimmune serum to inhibit at much lower concentrations. Nevertheless, negative results in such a system do not provide conclusive evidence for the absence of blocking factors. Besides the limitation of concentration there are a number of reasons why serum blocking factors may not be detectable in MLC.

- (a) They may be unable to inhibit initiator cell function in vitro.
- (b) They may be unstable.
- (c) They may be removed or inactivated by other cells, e.g., macrophages, before they can act on their targets.
- (d) Antiblocking substances are present in the culture, e.g., excess free antigen.

To overcome some of these objections two different approaches were used. One was to examine whether unresponsive serum might inhibit the generation of effector cells in MLC. The other was to test whether such serum would inhibit effector cell function in vitro. The latter system also has the advantage of permitting the use of much higher concentrations of mouse serum (see Chapter 4, Section 4).

The results of attempts to inhibit the generation of effector cells are shown in Table 59. At no concentration that was tested, up to the maximum of 5%, did unresponsive serum have any detectable effect on the production of effector cells in MLC.

Table 59. Attempts to inhibit the in vitro generation of effector cells with unresponsive serum.

Expt.	Effector cells	Serum in MLC	Serum in CML	% cytotoxicity for:	
				EL 4	P815
1	"Unresponsive"	2.0% control	none	34 ± 0.5	-
	"Unresponsive"	2.0% unresponsive	none	42 ± 2	-
	"Unresponsive"	2.0% unresponsive	11.0% control	31 ± 2	-
	"Unresponsive"	2.0% unresponsive	11.0% unresponsive	35 ± 2	-
2	"Unresponsive"	1.5% control	none	-	70 ± 1
	"Unresponsive"	1.5% unresponsive	none	-	64 ± 1
	"Unresponsive"	5.0% control	none	-	29 ± 1
	"Unresponsive"	5.0% unresponsive	none	-	35 ± 1

Lymph nodes and blood were taken from pools of 3 unresponsive mice bearing perfect C57B1 (expt. 1) or DBA/2 (expt. 2) skin grafts for $2\frac{1}{2}$ - 3 months and 2 control mice. Cell suspensions were cultured in MLC with irradiated CBA and C57B1 (or DBA/2) spleen cells, freshly prepared serum being added at the start of culture at the concentrations shown. After $4\frac{1}{2}$ days the cells were harvested and recultured with Cr-labelled target cells of the appropriate genotype at an effector:target cell ratio of 50:1 for 7 hrs (expt. 1) or 8 hrs (expt. 2). To some of the cytotoxic assay mixtures in experiment 1, unresponsive or control serum (which had been stored at -20°C during MLC sensitisation) was added to a concentration of 11%.

Even when 11% unresponsive serum was additionally present in the cytotoxic phase no inhibition of target cell killing was found. It must, however, be stressed again that the concentrations of mouse serum that could be used in the MLC phase were limited.

Attempts to inhibit the action of cytotoxic cells in the Cr-release test are shown in Table 60. No inhibition of killing by control sensitised cells occurred in the presence of 2.5% or 11% unresponsive serum. Nor could sensitised "unresponsive" cells be inhibited by 11% unresponsive serum. At both these concentrations, hyperimmune serum caused very strong inhibition of target cell destruction. However, when the concentration of unresponsive serum was raised to 20% (experiment 3 of Table 60) a highly significant inhibition of killing by immune cells was found. In the same experiment, serum prepared from neonatally tolerant mice failed to cause inhibition, while hyperimmune serum once again produced strong blocking. Tests on other pools of unresponsive serum have not yet been possible, so for the moment the conclusion that inhibitory factors are present in unresponsive serum must be regarded as preliminary.

Discussion.

These studies, together with those performed by other members of this department, have shown that mice made unresponsive in adult life by means of antigen pretreatment and a short course of ALS after grafting contain antigen-reactive cells and that a mechanism of active suppression maintains the unresponsive state for very long periods. This suppression seems to act mainly, though not necessarily exclusively, at the initiation level of the immune response rather than on effector cells, but beyond

Table 60. Attempts to inhibit killing by cytotoxic cells with serum from unresponsive mice.

Expt	Effector cells	Serum	% cytotoxicity for:			% Inhibition	
			EL4 ¹	P815 ²	A ³		
1 & 2	Unresponsive	0	0 ± 1	0 ± 1	-	-	-
	Control	0	24 ± 1	47 ± 1	-	-	-
	Unresponsive	0	13 ± 1	46 ± 1	-	-	-
	Control	2.5% control	19 ± 1	36 ± 1	-	-	-
	Control	2.5% unresponsive	18 ± 1	41 ± 1	-	5%	-14%
	Control	2.5% hyperimmune	5 ± 0.5	ND	-	74%	ND
	Control	11% control	11 ± 1	29 ± 1	-	-	-
	Control	11% unresponsive	13 ± 1	24 ± 2	-	-18%	17%
	Control	11% hyperimmune	1 ± 0.5	ND	-	90%	ND
	Unresponsive	11% control	8 ± 2	ND	-	-	-
	Unresponsive	11% unresponsive	8 ± 1	ND	-	0%	ND
3	Normal	20% normal	-	-	62 ± 2	-	-
	Normal	20% tolerant	-	-	61 ± 2	2 ± 4%	NS
	Normal	20% normal	-	-	65 ± 1	-	-
	Normal	20% unresponsive	-	-	44 ± 2	33 ± 3%	***
	Normal	20% hyperimmune	-	-	14 ± 2	78 ± 3%	***

Experiments 1 and 2 shown here were parts of experiments 1 and 2 described in Table 59. In experiment 1, serum which had been stored at -20° in sealed ampoules during the MLC sensitisation of cells was added to cytotoxic cultures at the concentrations shown. In experiment 2 serum added to the cytotoxic mixtures was freshly obtained from 1 unresponsive mouse and 1 control mouse belonging to the same group of mice which provided cell donors for MLC sensitisation (See Table 59).

In experiment 3, lymph node cells from normal mice were cultured in MLC with both irradiated CBA and irradiated A strain lymph node cells. After 41 days cells were harvested from MLC and recultured with Cr-labelled A strain lymphoma cells at a 125:1 effector:target cell ratio in the presence of 20% mouse serum freshly obtained from the following mice (2 mice in each group): mice made tolerant neonatally to A strain antigens and bearing perfect skin grafts for 4 weeks; normal mice matched for age and sex with these; mice made unresponsive in adult life to A strain antigens, bearing perfect skin grafts for 4 months; age/sex-matched controls for these. The cytotoxic reaction was stopped after 6 hrs.

In experiments 1 and 3 hyperimmune serum of the appropriate specificity was added as a positive control.

this it is not possible as yet to state with any certainty how the suppression operates.

The presence of small quantities of IgG₁ antibodies in these mice might suggest that the suppression is mediated by antibodies or antigen-antibody complexes. Indeed, for enhancement of rat kidney allografts, very small quantities of passively transferred antibody have been found highly effective in mediating this unresponsive state (533). As enhancement can also be produced in the rat following pre-immunisation (457), the possibility that the pretreatment of mice with antigen extract induces enhancing antibodies must be taken seriously, especially as in a combination differing only at non-H2 loci antigen pretreatment alone (without ALS or pertussis administration) caused unresponsiveness (525). Interestingly, in a tumour system, Chantler and Batchelor (534) reported that enhancement was optimal when lyophilised tumour extracts were administered about 2 weeks prior to inoculation of tumour cells; in the system described here, antigen extract is most effective when given 16 days before grafting (525). Very recently it has been reported that a KCl extract of EL 4 cells, optimally given 21 days prior to grafting, specifically stimulated the formation of antibodies able to block cell-mediated cytotoxicity in vitro and caused prolonged survival of C57 skin on Balb/c mice (535).

The inhibition of cytotoxicity with 20% unresponsive serum, while requiring confirmation, supports the idea of an inhibitory serum factor. The fact that unresponsive serum did not inhibit MLC cannot be construed as evidence that the block in these animals is primarily at the effector cell level, since quite possibly the same or other factors would be able to suppress MLC

reactions if it were possible to perform these with higher concentrations of mouse serum. As to the nature of the substance inhibiting the cytotoxic reaction, the strong inhibition of such reactions by hyperimmune serum, which contains predominantly IgG₁ antibodies (526), suggests that the small amounts of IgG₁ antibody in unresponsive serum may have been responsible.

There is therefore a case to be made out for the participation of antibodies. However, attempts to suppress reactivity *in vivo* against donor antigens with serum taken from unresponsive mice have so far failed (536). Furthermore, neither serum taken from mice 16 days after antigen pretreatment (the time when they would normally have been grafted and treated with ALS), nor hyperimmune serum was able to prolong graft survival when repeatedly injected into ALS-treated recipients (518).

The results of other experiments suggest that suppressor cells may play a role in maintaining the unresponsiveness. Thus, as mentioned above, spleen cells from unresponsive mice can transfer unresponsiveness to ALS-treated mice. Treatment of spleen cells with anti- θ abolished this effect (526). The apparent role of suppressor T cells thus parallels the findings in some other systems where unresponsiveness appears to be mediated by a T cell population (480, 537). On the other hand, the possibility that primed helper T cells are being transferred which can cooperate with host B cells to produce immunosuppressive antibody, or antibody to form antigen-antibody complexes, must be borne in mind. Furthermore, the evidence that suppressor T cells are important does not rule out a role for serum-borne factors - presumably suppressor cells are triggered by some signal, which could be

antigen-antibody complexes, and suppressor T cells may act by releasing an antigen-specific suppressive factor which could be a complex containing T cell receptor immunoglobulin.

Clearly much more work is required in this and other systems in order to unravel the apparent complexities of immuno-regulation. Although there are at present many diverse models of specific immunological unresponsiveness to allografts induced in adult animals it may well turn out that a common mechanism plays an important role in many of them. Thus, administration of enhancing antibody or pretreatment with antigen followed by immunosuppression (which, at least in cases where ALS is used, selectively depresses the cellular response (538, 539) and allows the production of some humoral antibody) may block the initial destructive phase of the immune response for long enough to allow the development of the regulatory phase, the latter being triggered by the presence of antigen-antibody complexes. The regulatory system, which in a number of systems seems to be based on suppressor T cells, would then be able to maintain graft survival in the absence of therapeutic agents, especially if continuously stimulated by low concentrations of antigen-antibody complexes.

Unfortunately, in man it appears that the survival of established kidney grafts is dependent on continuous immunosuppression, as complete withdrawal of drug therapy is known to lead to graft rejection. It could be that the drugs most commonly used for preventing graft rejection in man inhibit both destructive and regulatory components of the immune response, but the possibility cannot be ruled out that there are differences between man and

rodents in the manner in which the immune response is regulated. Nevertheless, the understanding of immunoregulation obtainable from a study of animal models should provide valuable guidelines for the manipulation of the immune response in man.

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ATTEMPTS TO DEMONSTRATE AN IN VIVO ROLE FOR SERUM BLOCKING FACTORS IN TOLERANT MICE¹

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SUMMARY

Six different in vivo situations were studied with a view to demonstrating the existence of blocking factors in the serum of mice made tolerant by neonatal inoculation of hybrid spleen cells: (1) graft-versus-host (GVH) reactivity of tolerant spleen cells; (2) inhibition of GVH reactivity of normal cells by serum from tolerant mice; (3) neonatal tolerance induction with suboptimal doses of hybrid spleen cells in the presence of serum from tolerant mice; (4) prolongation of skin allograft survival by passive transfer of tolerant serum; (5) transfer of tolerance to normal mice by parabiosis to tolerant animals; (6) breaking tolerance by adoptive transfer of normal spleen cells. The results of these experiments do not support the concept that blocking factors are primarily responsible for the induction and maintenance of neonatally induced tolerance. A hypothesis is proposed which might account for the apparent discrepancy between in vivo and in vitro investigations.

The widely held belief that experimentally induced tolerance to tissue allografts is attributable to a genuine failure of response, caused by the elimination or modification of cell clones, has been questioned. Thus, Voisin et al. (22) have shown that the serum of mice made tolerant at birth by the inoculation of viable lymphoid cells may be used to enhance the growth of a tumour of donor strain origin. More recently, Hellström et al. (13) have demonstrated that lymphoid cells from tolerant mice are to some extent cytotoxic in vitro to donor strain target cells, and that this cytotoxicity is specifically inhibited by serum from tolerant animals. Similar results have been obtained in tetraparental mice whose lymphoid cells are evidently reactive to parental strain target cells as measured by in vitro cytotoxicity (23) or the mixed lymphocyte culture (MLC) test (18), provided that serum from tetraparental mice is not present in the culture medium. It has therefore been suggested that the state of tolerance is caused by an active response leading to the formation of serum-borne factors (antibodies or antigen-antibody complexes?) that restrain the cells involved in

the allograft reaction. Although the mechanism might well be different, it could be analogous to that underlying the phenomenon of immunological enhancement. Some of the earlier experiments, both in vivo and in vitro, do not support such an interpretation (for review see reference 5). The experiments reported here represent an attempt to reveal an in vivo role for blocking factors, using six different approaches.

MATERIALS AND METHODS

The production of tolerant mice followed in every detail the method described by Hellström et al. (13), viz., CBA mice were injected i.p., within 24 hr of birth, with 5×10^7 (CBA $\times$ A)_F₁ spleen cells and their cells and sera were usually harvested 6-8 weeks later. Hellström et al. have shown, and we can confirm, that such mice develop a lasting unresponsiveness to strain A skin grafts almost without exception. Cells were used fresh in a phosphate-buffered Ringer's solution (pH 7.4) and viability counts were routinely carried out by trypan blue exclusion; suspensions with less than 70% viability were not used. Cell suspensions were washed twice in large volumes of medium. Unheated serum was used fresh whenever possible, but much of it was

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stored at -80°C . Donors providing control tissues or serum were selected to be of the same age and sex as the tolerant animals.

Tolerant mice did not have detectable haemagglutinin titres when tested by the method of Gorer and Mikulska (11), and haemagglutination inhibition tests (9) did not reveal the presence of strain A antigen in the serum of tolerant mice. Quantitative chimera tests (7) revealed that approximately 5% of their spleen cells were donor cells.

RESULTS

Are spleen cells from tolerant mice responsive to strain A antigens when removed from the constraint of the host environment? The ability of tolerant cells to cause splenomegaly following injection into neonatal $(\text{CBA} \times \text{A})\text{F}_1$ or strain A mice was tested, and Table 1 summarises the results. The reactivity of tolerant and normal cells was compared, F_1 cells providing the base line data. Each litter formed an internally controlled experiment, and body and spleen weights were established 8 days after injection of cells. The spleen index (SI) (20) was calculated as follows:

$$\text{SI} = \frac{\text{mean relative spleen weight of mice receiving test cells}}{\text{mean relative spleen weight of littermates receiving } \text{F}_1 \text{ cells}}$$

where relative spleen weight = spleen weight(mg)/100 g body wt. Hybrid litters received 10^7 cells i.p.; strain A litters were injected i.v. with 1.5×10^7 cells to ensure that tolerance to CBA antigens was adequately established (6).

In all 4 experiments, normal cells proved to be appreciably more reactive than cells from tolerant mice, and with one exception the spleen indices of the tolerant cell recipients approximated closely to unity. In litter 4 the index was 1.16 because one of three spleens was enlarged—an anomalous result which might have been caused by incomplete tolerance induction in the cell recipient, i.e., to an allograft response against the CBA cells.

These results confirm older data (12, 15) showing that tolerant cells are incapable of mounting GVH reactions.

Can serum from tolerant mice inhibit GVH reactivity of normal CBA spleen cells? Table 2 gives the results of experiments in which F_1 and strain A litters were injected, respectively, with 10^7 and 5×10^7 normal female CBA spleen cells, littermates having been inoculated with serum from either normal or tolerant donors. Serum (0.05 ml) was injected 2 hr prior to cell injection, and a further 0.1 ml was given 2 days later (litters 1-3).

No significant differences were found between the degree of splenomegaly in the two groups.

TABLE 1. GVH reactivity of spleen cells from CBA mice injected neonatally with 5×10^7 $(\text{CBA} \times \text{A})\text{F}_1$ spleen cells

Experiment No.	Host litter and No. of cells injected ^a	Cells injected	No. of mice	Mean spleen weight (mg/100 g body wt) $\pm$ SE	Spleen index
1	$(\text{CBA} \times \text{A})\text{F}_1$ (24-48); 10^7	F_1	2	565.1 $\pm$ 67.0	
		Tolerant CBA	3	602.7 $\pm$ 60.4 ^b	1.07 ^b
		Normal CBA	2	786.4 $\pm$ 9.9	1.39
2	$(\text{CBA} \times \text{A})\text{F}_1$ (<24); 10^7	F_1	1	842.5	
		Tolerant CBA	2	839.6	1.00
		Normal CBA	1	1,237.4	1.47
3	A strain (<24); 1.5×10^7	F_1	2	846.6 $\pm$ 13.4	
		Tolerant CBA	3	979.5 $\pm$ 90.6 ^c	1.16 ^c
		Normal CBA	2	1,453.8 $\pm$ 16.2	1.72
4	A strain (<24); 1.5×10^7	F_1	2	843.7 $\pm$ 69.2	
		Tolerant CBA	1	834.1	0.99
		Normal CBA	3	1,295.0 $\pm$ 147.0	1.53

^a Numbers in parentheses indicate age (hr) of host. Hybrid litters were injected i.p. and A strain litters were injected i.v. Test cells were injected into neonatal littermates for splenomegaly assay on day 8.

^b One of three spleens was somewhat enlarged.

^c One of three spleens was enlarged.

TABLE 2. Attempt to inhibit the GVH reactivity of normal CBA spleen cells with tolerant serum from CBA mice injected neonatally with 5×10^7 (CBA $\times$ A) F_1 cells

Litter No.	Host strain and No. of cells injected ^a	Serum ^b	No. of mice	Mean spleen weight (mg/100 g body wt) $\pm$ SE	Normal: tolerant ratio	SE ^c
1	(CBA $\times$ A) F_1 (newborn); 10^7	Normal	4	1,049.3 $\pm$ 45.8	0.85	0.06
		Tolerant	4	1,227.5 $\pm$ 60.5		
2	A strain (newborn); 5×10^7	Normal	2	1,434.1 $\pm$ 142.3	1.04	0.11
		Tolerant	3	1,376.7 $\pm$ 57.7		
3	A strain (newborn); 5×10^7	Normal	3	1,366.7 $\pm$ 33.1	0.94	—
		Tolerant	1	1,460.9		
	(CBA $\times$ A) F_1 (36-48); 10^7	Normal	4	1,336.4 $\pm$ 52.6	1.10	0.07
		Tolerant	3	1,215.5 $\pm$ 64.3		

^a Cell suspensions were injected i.p. into littermates; splenomegaly assay followed 8 days later. Numbers in parentheses indicate age (hr) of host.

^b Litters 1-3 were injected i.p. with 0.05 ml of serum 2 hr prior to cell injection and with 0.1 ml 2 days later. Litter 4 received cells preincubated with 0.05 ml of serum/dose; cells and serum were injected together in a total volume of 0.1 ml. Mice were further injected with 0.1 ml of serum on days 1, 2, 3, 4, and 5.

^c Calculated from the variance of ratios (1).

TABLE 3. Attempt to prolong the survival of A strain skin grafts in CBA female mice by passive transfer of serum from tolerant CBA mice^a

Day	Scores (% epithelial survival) of grafts of serum recipients	
	Normal	Tolerant
9	90, 25, Trace, 0, 0	90, 50, 50, Trace, 0
10	50, Trace, 0	75, 25, Trace, 0
11	Trace, 0	50, Trace
12	0	Trace, 0

^a All mice were injected with 0.5 ml of serum on day of grafting and on day 1, followed by 0.2 ml on days 3, 5, and 7. Tolerant serum was from 8-week-old mice injected at birth with 5×10^7 (CBA $\times$ A) F_1 spleen cells.

This experiment was repeated with a very much higher dose of serum (Table 2, litter 4); in addition, the cells were preincubated for 30 min at room temperature in medium containing 50% of the appropriate serum. The suspension, 0.1 ml containing 0.05 ml of serum, was injected i.p. 24-36 hr after birth, and 0.1 ml of serum was injected daily for 5 days. The assay was performed on the 8th day after cell injection, and the relative spleen weight ratio of normal:tolerant serum recipients again did not depart significantly from unity.

Will serum from tolerant mice transferred passively prolong skin allograft survival? Skin allografts have, in the past, been found to be

peculiarly resistant to the action of enhancing antibodies (8). Nevertheless, if blocking antibodies or antigen-antibody complexes are involved in tolerance, one could reasonably expect the transfer of tolerant serum to extend the survival of skin allografts in normal recipients. Two groups of 5 CBA females were grafted with strain A tailskin and injected i.p. immediately after transplantation with 0.5 ml of normal or tolerant serum. A second dose of 0.5 ml was given on the following day, and thereafter 0.2 ml was injected on days 3, 5, and 7. The grafts were first inspected on day 9 and daily thereafter.

It is clear from Table 3 that there was no discernible difference in the rejection pattern of the two groups.

Will serum from tolerant mice facilitate induction of tolerance in neonatal mice? Tolerance induction in neonatal mice is strictly dose-dependent (6); it is age-dependent, too (6), and the important events leading to the establishment of unresponsiveness must therefore occur within a few days of birth. Newborn mice were injected with a dose of F_1 spleen cells (2.5×10^6) that was known to produce tolerance in only a fraction of recipients. The injection of tolerant serum (0.05 ml) into these mice immediately after cell inoculation and on the following day can therefore be expected to raise the incidence of tolerant animals compared with a normal serum control group. This expectation was unfulfilled (Table 4).

Is tolerance transferable from tolerant to normal mice by parabiosis? Martinez et al. (14) carried out experiments which showed that a high proportion of normal Z (C3H) mice became tolerant of Ce skin grafts after 2 weeks of parabiosis with Z mice tolerant of Ce histocompatibility antigens. They interpreted this finding in terms of tolerance induction by Ce cells circulating in the blood of the tolerant partners—an explanation that seems highly probable in view of the fact that the two strains were not only H-2-compatible but differed at only a single H locus (R. A. Good, personal communication). Nevertheless, as Voisin has indicated in his recent review (21), this finding is now open to the interpretation that blocking factors transferred from the tolerant to the normal partners played a role. Parabiosis experiments were therefore undertaken to establish whether transfer of unresponsiveness occurs in an H-2-incompatible situation, even if only transiently, following the termination of parabiosis.

Tolerant CBA mice were parabiosed with normal CBA mice of the same sex, age, and weight, using the coelomic method of Sauerbruch and Heyde (19). The tolerant mice were either 8 weeks old (not grafted) or about 4 months old (having received strain A grafts 2 months before). That there was satisfactory blood interchange was demonstrated by the injection on day 9 of ⁵¹Cr-labeled syngeneic red blood cells, free of white cells, into a few of the tolerant

TABLE 4. Attempt to facilitate tolerance induction in newborn mice with serum from tolerant CBA mice^a

Postoperative day	Surviving grafts (%)	
	Normal serum	Tolerant serum ^b
11	86	100
23	57	60
30	57	46
36	50	46
44	50	33
90	50	33

^a CBA mice were injected i.v. with 2.5×10^6 (CBA $\times$ A)_F₁ spleen cells within 24 hr of birth and i.p. with 0.05 ml of serum immediately after as well as on the following day. All mice (15/group) were grafted 6 weeks after birth.

^b Tolerant serum was obtained from 16-day-old CBA mice injected neonatally with 5×10^7 (CBA $\times$ A)_F₁ spleen cells.

TABLE 5. The survival of skin allografts in tolerant and normal CBA mice after 15 days of parabiosis

Pair No.	Parabionts ^a	Sex	Graft survival time (days)
1	Normal	Male	12
	Normal	Male	13
2	Normal ^b	Male	11
3	Normal ^b	Male	14
4	Normal ^b	Female	14
5	Normal	Male	8
	Tolerant	Male	<7
6	Normal	Male	7
	Tolerant	Male	<7
7	Normal	Female	7
	Tolerant	Female	<7
8	Normal	Female	7
	Tolerant	Female	10
9	Normal ^c	Female	<7
	Tolerant	Female	<7
10	Normal ^c	Female	9
	Tolerant	Female	8
11	Normal	Male	<9
	Tolerant ^d	Male	10
12	Normal	Male	<9
	Tolerant ^d	Male	<9
13	Normal	Female	<9
	Tolerant ^d	Female	10
14	Normal	Female	<9
	Tolerant ^d	Female	<9

^a Tolerant mice were injected i.p. at birth with 5×10^7 (CBA $\times$ A)_F₁ spleen cells; parabiosis began 6 weeks after birth. Skin was transplanted on day of separation unless otherwise stated.

^b Partner died during or after separation and grafting.

^c Injected on day of parabiosis with 0.5 ml of tolerant serum.

^d Skin was transplanted 2 months before parabiosis; survival times refer to day of separation.

partners. By sampling the blood of the normal partners and counting the samples in a liquid scintillation counter, it was found that about 50% equilibration occurred within as short a time as 30 min. Parabiosis was terminated after 15 days and all mice, except those tolerant mice already carrying skin grafts, immediately received strain A grafts. Freshly transplanted grafts were first inspected on day 7 or 9.

The results were unequivocal (Table 5). None of the normal partners showed even the most ephemeral unresponsiveness; on the contrary,

they had evidently been sensitised, either because F_1 donor cells circulating in the blood of the tolerant partners had stimulated their lymphoid organs and/or because peripatetic lymphocytes from the normal partners were stimulated by F_1 cells present in the tissues of the tolerant mice. The first of these possibilities could, to some extent, invalidate the experiment, for it could be argued that sensitisation had occurred before an adequate level of blocking factors had built up in the normal animals. In order to meet this criticism, 2 normal mice joined to tolerant partners were injected on the day of parabiosis with 0.5 ml of tolerant serum (Table 5, pairs 9 and 10). The results were the same.

Conversely, tolerance was always promptly broken in the tolerant partners, and quantitative chimera tests indicated that they no longer possessed F_1 hybrid donor cells.

Can tolerance be broken by normal syngeneic spleen cells? Relevant to the parabiosis experiments is our observation that i.p. transfer of 10^8 normal female CBA spleen cells to tolerant mice led to rejection of well established grafts in 12 of 12 cases, rejection occurring 3–4 weeks after adoptive transfer. The quantitative aspects of this approach are being studied further; but rejection of grafts after a latent period of about 3 weeks indicates that the transferred cells had not come under the effective influence of blocking factors.²

DISCUSSION

All experimental models used to investigate the *in vivo* role of blocking factors in tolerant mice have given negative results. We are well aware that negative results are frequently open to technical objections; for example, it could be argued that the attempt to passively transfer unresponsiveness to adult mice would have succeeded if larger amounts of serum had been used. All the same, if blocking factors play as important a role as has been suggested (13), one or other of these approaches should have given a positive indication. We attach particular importance to our failure to modify the behaviour of relatively small numbers of lymphoid cells in a neonatal GVH assay, and to our inability to

enhance tolerance induction in neonatal mice, for in both situations the amounts of serum injected represent a high proportion of the hosts' own plasma volumes. Further, it is in the neonatal and early postnatal mouse that the events leading to tolerance must occur following the injection of allogeneic cells.

It might be objected that the cells from tolerant mice synthesised blocking factors after transfer and thus interfered with their own cell-mediated response. This seems highly improbable, for it would imply a degree of sensitivity to blocking factors that does not appear to exist in *in vitro* experiments (13, 18, 23). Atkins and Ford (2) have reexamined the reactivity of rat thoracic duct lymphocytes using the sensitive popliteal lymph node assay. Not only did cells from tolerant hosts fail to give a response, but normal cells remained fully reactive when injected into the footpads of F_1 rats together with a large excess of tolerant cells. In their experiments the possibility of blocking factors being produced by tolerant cells after transfer was therefore formally excluded.

The sera used by us came from mice which were in every sense identical to those used by Hellström et al. in their *in vitro* demonstration of blocking factors, and we have therefore refrained from using the microcytotoxic assay. MLC experiments are in progress and preliminary results indicate that tolerant cells are not stimulated to nearly the same extent as normal cells; we do not yet know, however, whether an MLC reaction by normal cells can be inhibited with tolerant serum.

Taken together with previously reported data (5) our results show that the biological role of blocking factors is far from clear and that it would be premature indeed to abandon the concept of tolerance as a central failure of responsiveness (3). The apparent contradiction between the *in vivo* and *in vitro* data is not easy to reconcile, but it could perhaps be done in the following manner. Since bone marrow-derived (B) cells are known to be more resistant to tolerance induction with certain proteins (10, 16) and haptens (17) than thymus-derived (T) cells, and since humoral antibodies are not the mediators of the reaction against skin allografts (4), it is quite possible that T cell tolerance and antibody production by B cells can coexist in a mouse made unresponsive by neonatal inocula-

² These results are strongly supported by Billingham et al. (3a) who broke tolerance with as few as 15×10^6 blood leucocytes.

tion of allogeneic cells. To explain the main findings it would be necessary to postulate the existence of a subpopulation of antigen-sensitive cells (TR) that are resistant to tolerance induction but susceptible to the action of blocking factors. If this subpopulation represents a very small fraction of the total number of antigen-sensitive T cells, the majority (TT) having become tolerant, it might not be adequate to register in the kind of in vivo experiments described here, while in assays in vitro—especially in those in which a great excess of effector cells to target cells is required and in which lymphokine factors may be involved—it could lead to the statistically significant but nevertheless rather undramatic cytotoxicity described by Hellström et al. (13). Both classical tolerance and blocking factors could thus play a role in tolerance induction and the prevention of autoimmunity. Unfortunately, this suggestion does not explain satisfactorily why some workers (5, 24) have found that, even in vitro, tolerant cells are not stimulated by donor strain target cells.

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In Vitro Reactivity of Lymphoid Cells From Tolerant Mice

By P. C. L. Beverley, L. Brent, C. Brooks, P. B. Medawar, and E. Simpson

THIS paper examines the contention of Hellström, Hellström, and Allison¹ that mice made tolerant by neonatal injection of allogeneic cells do in reality contain immunologically activated lymphocytes which are prevented from attacking what would otherwise be their target by the interposition of a serum factor. Tolerance would thus be due to a peripheral interference with the immune process and not to a central failure of response. The experiments described here differ in principle from those of Hellström et al. in that (1) different degrees of tolerance were designedly produced by the neonatal inoculation of varying numbers of cells; and (2) mice intended to be fully tolerant were shown to be so by skin grafting and by the mixed lymphocyte culture (MLC) reaction. A further important departure was that mice belonging to each dosage group were appraised for chimerism by *in vivo* tests carried out on a scale sufficient to reveal *degrees* of chimerism. For the background and history of the idea that tolerance is due to peripheral interference with an immune response see Brent² and Medawar (in this issue).

METHODS

Subjects

The mice used were sublines of strains CBA, A/Jax, and C57BL maintained at St. Mary's and

hybrids between them [(CBA × A), (CBA × C57BL)].

Procedure

CBA mice received intraperitoneal injections of 50, 25, 12.5, or 5×10^6 (CBA × A)_{F₁} spleen cells within 24 hr of birth. Mice in which an assurance of complete tolerance was required were skin grafted at 6–8 wk and none were accepted for experimental tests unless their grafts had survived unblemished for at least 30 days. For the groups injected with lower cell doses the degree of tolerance to skin grafts was established in littermates of the mice used in the tests. The percentage of mice made fully tolerant at each dose level is entered in Table 1, Column 2.

Chimerism Tests

A quantified variant of the technique of Mitchison³ was used. Evidence for chimerism was sought in the spleens of mice whose lymph nodes were tested for cytotoxicity. Each mouse was tested individually. Chimerism tests took the form of injecting spleen cells into normal CBA mice (secondary hosts) which were grafted 3–4 days later with strain A skin. If the spleens contained cells of A specificity the secondary hosts would be expected to reject the test grafts in accelerated fashion. Three dose levels, viz, 10^7 , 10^6 , and 10^5 , were used. Positive results at the lower dose levels would indicate a high degree of chimerism. An estimate of the per cent of donor cells present in each mouse (see Table 1) was made by comparing the level of sensitivity with that produced by cell mixtures containing known numbers of F₁ cells.

Media

The following abbreviations are used in the text: FCS = fetal calf serum, MEM = Eagle's minimal essential medium, BSS = balanced salt solution, CM = equal mixture of MEM and RPMI 1640, buffered with bicarbonate/CO₂, PBS = phosphate-buffered saline.

Mixed Lymphocyte Cultures

Our procedure was that of Festenstein,⁴ with minor variants. Lymph node (LN) cells were washed three times in 20-ml PBS containing 2% FCS and finally suspended in CM with 10% FCS

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to give the desired concentration of LN cells and red cells. Equal vols of the control suspensions were mixed to provide mixed cultures.

For labeling, 20 μ l of a 1.6×10^{-4} M solution of tritiated thymidine (specific activity 30 μ Ci/ μ mole) were added to the 0.5 ml cultures. 16 hr later cells were harvested and prepared for scintillation counting. After washing the cells part of each CBA cell culture was fixed in glutaraldehyde for cell counts.

The results are expressed as the degree of stimulation in mixed cultures per 10^6 responder-strain cells viable at the end of the labeling period. The degree of stimulation is calculated by subtracting the mean count/min (cpm) in control tubes from the cpm of mixed culture tubes. In the experiment shown in Fig. 1 the cells were washed three times; washing five times produced the same result.

Cytotoxic Tests

These were carried out with Falcon 3040 micro-test II plates. Target cells were strain A fetal fibroblasts dissociated by trypsinisation and main-

tained in culture for 1-2 wk (circa 3 passages). For use as targets the fibroblasts were dispensed in 200 μ l vol (circa 1000 cells) into the wells and incubated 12-18 hr for adhesion (plating efficiency circa 10%). They were then exposed to 2000 rad gamma radiation in a ^{60}Co source and used as targets 3-4 hr after. Lymphoid cells were prepared by teasing out lymph nodes in BSS, washing once in BSS once in 20% FCS/MEM, and suspending at 2×10^6 /ml in 20% FCS/MEM. Sera were diluted 1:6 in MEM. Cytotoxicity tests were performed by preincubating target cells with diluted serum for 45 min, followed by replacement of serum with lymphoid cells. The plates were incubated for 48 hr at 37°, washed three times to remove lymphocytes, fixed, stained and counted. The net ratio of lymphoid to target cells was 4000:1 as in the work of Hellström et al.

Comments on Techniques

The use of fetal as opposed to adult fibroblasts as targets was unobjectionable because (1) isotopic antiglobulin tests using anti-H-2 sera

Table 1. Test for Donor Cell Chimerism: Median Survival Times (MST's) of Strain A Grafts Transplanted to CBA Males 3-4 Days After Intraperitoneal Inoculation of Varying Doses (10^7 , 10^6 , 10^5) of Spleen Cells Obtained From CBA Donors That Had Been Injected Intraperitoneal Neonatally with 50, 25, 12.5 or 5×10^6 (CBA $\times$ A) F_1 Spleen Cells

F_1 Cells → Donors ($\times 10^6$)	(No.) Donor Mice Tolerant* (%)	Donors (No.)	Cells Recipients (No.)	Total Recipients (No.)	MST's† (Days)	Approximate F_1 Cells in Donors‡ (%)
0	0	3	10^7	10	$10^{1/2}$	0
			10^7	ND	ND	
50	97	3	10^6	21	$9^{1/2}$, $7^{1/2}$, < 7	> 10, >> 10, >> 10
			10^5	23	8, 7, 7	
			10^7	25	$7^{1/2}$, 7, 7, 7, 7	
25	62	6	10^6	40	$10^{1/2}$, 8, 11, $8^{1/2}$, $11^{1/2}$, 10	1, 5, 1, 4, 1, 3
			10^5	40	10, 10, 10, 10, 11, 10	
			10^7	23	< 7, < 7, $9^{1/2}$, < 7, ND	
12.5	40	5	10^6	35	8, $9^{1/2}$, 10, $10^{1/2}$, $10^{1/2}$	5, 2, 0.5, < 1, < 10
			10^5	35	10, $10^{1/2}$, $10^{1/2}$, $10^{1/2}$, 10	
			10^7	33	$10^{1/2}$, < 7, $10^{1/2}$, 7, $10^{1/2}$, < 7	
5	0	6	10^6	43	10, 10, $10^{1/2}$, 9, $10^{1/2}$, 9	< 0.1, 1, < 0.1, 1, < 0.1, 1
			10^5	41	$9^{1/2}$, 10, $10^{1/2}$, $10^{1/2}$, 11, 11	

*This figure was obtained from groups of ten mice injected concomitantly with the actual cell donors. The figures represent the per cent of grafts without signs of rejection 75 days following transplantation.

†The MST's of grafts are recorded here for individual mice at the three cell doses used in the chimera tests. The first figure for each cell dose refers to the same donor etc.

‡Calculated by comparing the MST's with those obtained from mice injected with mixtures of known numbers of F_1 and syngeneic cells.

ND, no data.

showed a representation of strain A antigens; and (2) the cells were, in fact, vulnerable to sensitized lymphoid cells: in Table 2, contrast the entries in line "ILN + CS" (immune lymphocytes + control serum) with the entries for "CLN + CS" (control lymphocytes + control serum). Target cells were irradiated in order to abolish a tire-some 'feeder effect' detected in earlier experiments, in which the number of target cells sometimes greatly increased during cultivation. Mice belonging to the lower dosage groups were not skin-grafted before cytotoxicity tests. Most mice of the 50×10^6 dosage group were grafted but others were not; both groups gave identical results.

RESULTS

Chimerism tests: The results of the tests for degrees of chimerism are shown in detail in Table 1 and in summary form in Table 3.

MLC tests: Eight individual mice and two cell pools from a number of donors were tested. Figure 1 illustrates a typical experiment made upon two CBA mice which had received 50×10^6 (CBA $\times$ A) F_1 spleen cells at birth. Their cells responded to third party (CBA $\times$ C57BL) F_1 targets but not to (CBA $\times$ A) F_1 cells.

Cytotoxicity tests: Table 2 records the results of a representative experiment from each of four groups of CBA mice given different doses of F_1 spleen cells at birth. The top entry in each column represents the baseline, i.e., the number of cells surviving exposure to normal syngeneic lymphoid cells in the presence of normal serum. In all experiments the exposure of target cells to deliberately sensitized lymphoid cells (ILN) in the presence of normal serum caused a significant diminuation of their number. Cells from mice that had received 50×10^6 donor cells had no such effect; nor did serum from these mice impede the attack of sensitized cells upon their targets. (The relevant entries are "ILN + TOL 5" from specified sources.) Cytotoxic cells were demonstrable only in non-

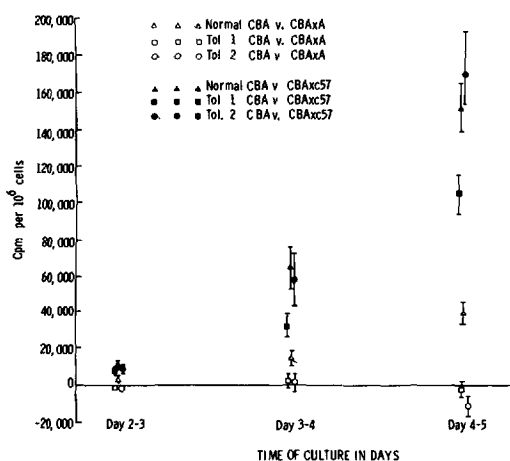


Fig. 1. MLC response of lymph node cells from CBA mice tolerant to strain A skin grafts against (CBA $\times$ A) F_1 and (CBA $\times$ C57BL) F_1 lymph node cells. Each point is based on the mean of five tubes. Bars indicate standard error of mean.

tolerant mice. The only circumstance under which a blocking effect could be discerned was that in which the neonatal cell inoculum had not been sufficient to induce complete tolerance with respect to skin grafts but yet led to a low (and probably transient) degree of chimerism. Orthodox allo-immune serum had no blocking power.

DISCUSSION AND CONCLUSIONS

Results summarized in Table 3 show that CBA mice which had received neonatal injections of 50×10^6 F_1 cells are tolerant and strongly chimeric; their lymph node cells had no cytotoxic activity and their serum did not interfere with the cytotoxicity of deliberately sensitized cells. At the other extreme, mice that had received 5×10^6 spleen cells at birth were only marginally or not at all chimeric, they were nontolerant, and 4/12 contained cytotoxic lymphoid cells. Their sera did not block the activity of presensitized cells. Perhaps the most interesting results—and those that may account for the discrepancy between

Table 2. Results of One Experiment from Each Group of CBA Mice Given Different Doses of (CBA $\times$ A) F_1 Cells at Birth

Source of Lymphocytes + Relevant Serum	Number of F_1 Cells Inoculated at Birth			
	50-	25	12.5	5
CLN + CS	68.33 $\pm$ 5.30	42.83 $\pm$ 6.09	51.17 $\pm$ 6.36	284.33 $\pm$ 9.22
Tol 1 LN + CS	(93.50 $\pm$ 10.18	(62.50 $\pm$ 4.23	(35.17 $\pm$ 3.96	(246.00 $\pm$ 18.67
Tol 1 LN + Tol 1 S	(65.83 $\pm$ 7.32	(48.83 $\pm$ 2.99	(41.50 $\pm$ 4.54	(250.83 $\pm$ 14.23
Tol 2 LN + CS	(95.17 $\pm$ 12.84	(69.00 $\pm$ 4.59	(49.00 $\pm$ 2.48	(211.00 $\pm$ 7.45***
Tol 2 LN + Tol 2 S	(87.17 $\pm$ 7.66	(57.50 $\pm$ 12.25	(37.00 $\pm$ 5.47	(227.67 $\pm$ 6.96***
Tol 3 LN + CS	(70.00 $\pm$ 5.47	(64.33 $\pm$ 4.01	(50.83 $\pm$ 5.93	(215.17 $\pm$ 8.56***
Tol 3 LN + Tol 3 S	(87.17 $\pm$ 4.92	(49.67 $\pm$ 3.30	(54.17 $\pm$ 9.19	** (261.83 $\pm$ 8.37
Tol 4 LN + CS			(35.17 $\pm$ 3.59	(228.83 $\pm$ 7.08***
Tol 4 LN + Tol 4 S			(38.17 $\pm$ 3.68	(190.67 $\pm$ 8.97***
ILN + CS	25.00 $\pm$ 2.61***	29.50 $\pm$ 2.16*	20.33 $\pm$ 2.16***	214.83 $\pm$ 7.10***
ILN + Tol 1 S	13.50 $\pm$ 1.88***	48.33 $\pm$ 3.07+++	36.50 $\pm$ 5.25+	176.17 $\pm$ 10.92***
ILN + Tol 2 S	12.17 $\pm$ 2.15***	31.17 $\pm$ 2.21	32.00 $\pm$ 5.99	191.33 $\pm$ 7.35***
ILN + Tol 3 S	17.17 $\pm$ 0.79***	28.17 $\pm$ 3.15	22.50 $\pm$ 3.47**	206.17 $\pm$ 4.21***
ILN + Tol 4 S			35.17 $\pm$ 1.92*+++	139.50 $\pm$ 4.67***
ILN + I serum	20.67 $\pm$ 2.25***	30.50 $\pm$ 2.35		177.67 $\pm$ 13.24***
Cytotoxic Cells	0/3	0/3	0/4	3/4
Blocking ILN	0/3	1/3	2/4	0/4

* = significant cytotoxicity compared with CLN + CS

+ = significant abrogation of ILN cytotoxicity

* (= significant abrogation of Tol LN + CS (cf. CLN + CS) cytotoxicity by Tol serum, LN, lympho node, S, serum, C, control, Tol LN/S, lymphocytes or serum from neonatally injected mice.

* or + = $0.01 < p < .05$

** or ++ = $0.001 < p < .01$

*** or +++ = $p < .001$

the data of Hellström et al. and our own—are those from mice that had received 25 or 12.5×10^6 cells. Many of these mice, especially from among the lower dose group, were only partially tolerant, and mice in both groups contained fewer donor cells than the 50×10^6 cell group. Two of eight (12.5 group) had cytotoxic cells at a marginal level of significance, and 5/11 (both groups) possessed serum factors that interfered with the cytotoxic activity of presensitized cells. It is therefore possible that the mice used by Hellström, Hellström, and Allison¹ were not fully tolerant. It is indeed unfortunate that the mice used in their tests and assumed by them to be tolerant (on the strength of skin graft tests or litter mates) were not shown to be so. At the time during which their experiments were carried out (1969/70) CBA mice at the National Institute for Medical Research were passing through a period of immunological hyperreactivity probably associated with the adoption of an autoclaved or irradiated diet during the institution of an SPF husbandry regimen—an interpretation now being investigated. It was at that time extremely difficult to produce tolerance by methods that had worked reliably in the past—e.g., neonatal injection and the combination of cell-free extracts with immunosuppressive agents.

The circumstances favoring the occurrence of blocking factors in our experiments seem to be those in which a low degree of tolerance is accompanied by a low level of chimerism. Inasmuch as chimerism signifies the persistence of donor antigen in the animals and alloantibody itself was ineffective (see above) it may be thought likely that the blocking factors are antigen-antibody complexes.

Table 3. Summary of Cytotoxicity Data, Chimerism Tests, and Skin Graft Survival From All Mice Tested

Number of F ₁ Cells at Birth ($\times 10^6$)	Cytotoxic Cells	Blocking ILN	Degree of Chimerism	Per Cent Skin Graft Survival (75 Days)
50	0/17	0/7	+++	97
25	0/7	1/3	++	62
12.5	0/8	4/8	++	40
5	4/12	0/12	±	0

Our in vitro data uphold the hypothesis⁵ that a central failure of the immunological response accounts for one form of specific unresponsiveness, and they receive strong support not only from numerous earlier studies² but also from recent in vivo experiments in mice,⁶ rats,⁷ and chickens.⁸

SUMMARY

(1) Neither cytotoxic cells, MLC-reactive cells nor blocking factors were found in CBA mice made demonstrably tolerant and chimeric at birth by the injection of 50×10^6 (CBA $\times$ A)F₁ spleen cells.

(2) Serum blocking factors but not cytotoxic cells were revealed in mice that had received 25×10^6 cells (4/8) and 12.5×10^6 (1/3). Such mice enjoy a relatively low level of chimerism and in both groups many were incompletely tolerant.

(3) Cytotoxic cells but not blocking factors were demonstrable in some of the mice injected with 5×10^6 F₁ cells—a dose insufficient to induce more than the most fleeting tolerance and marginal chimerism.

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