QUANTITATIVE STUDIES ON THE MIXED LYMPHOCYTE INTERACTION IN RATS

IV. Immunologic Potentiality of the Responding Cells*

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Recent studies have established the immunologic significance of the mixed lymphocyte interaction (MLI)[1]—the proliferative reaction which occurs when lymphocytes from genetically disparate donors are mixed and cultured together. Even though the activity of cells in this system is assessed in terms of proliferation rather than the elaboration of a specific immunologic product, it is apparent for several reasons that the responding cells in the MLI are immunologically competent lymphocytes which recognize and are triggered to react to specific histocompatibility isoantigens (1–7).

Comparative studies with isogenic strains of rats have pointed to the similar immunoproliferative behavior of lymphocytes in the MLI in vitro and in a localized graft-vs.-host (GVH) reaction in vivo (7). The stimulatory factors have been shown to be transplantation isoantigens determined by the major AgB histocompatibility locus in this species (6) and the cells that respond are those comprising the circulating lymphocyte pool (4). The immunogenetic status of the cell donors is an important determinant of the magnitude and specificity of the proliferative response: Cells from immunologically tolerant animals are specifically unreactive to those cellular isoantigens to which the donors have been rendered tolerant; cells from F_1 hybrid donors do not react against parental strain isoantigens; and cells from neonatally thymectomized donors display only a limited proliferative response (5, 8).

The proportion of parental strain rat lymphocytes which react to the cellular isoantigens of an F_1 donor has recently been shown to be unusually high—approximately 2% (9). A similar proportion responds to homologous host antigens in a GVH reaction by producing pocks on the avian chorioallantoic membrane or by inducing splenomegaly in neonatal murine hosts (10–12).

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[1] *Abbreviations used in this paper:* ASC's, antigen-sensitive cells; BN, Brown Norway rat strain; GVH, graft-vs.-host; H, histocompatibility; ^{3}H-TdR, tritiated thymidine; L, Lewis rat strain; MLI, mixed lymphocyte interaction; TCA, trichloroacetic acid; tol, tolerant; V, variable portion cistrons.

In view of the clonal selection hypothesis (13), this seems to be a surprisingly large number of lymphocytes with the capability of recognizing and responding to a given isoantigen system. The present studies were designed, therefore, to evaluate the following possibilities: (*a*) that the responding population of cells in the MLI might include a large component of nonspecifically activated lymphocytes "recruited" to proliferate by the activity of a small minority of presumably specifically reactive cells; (*b*) that the responsive population might be pluripotential and have the capacity to react to any one of several different histocompatibility antigen systems; or (*c*) that the reactive cells are unipotential and respond only to a particular antigenic determinant, but that the number of different antigen systems capable of provoking responses of such a magnitude is necessarily small.

The results provide evidence which favors the interpretation that the responding cells in the MLI are *not* recruited or activated in some nonspecific manner; that they are restricted in their response capacity, so that any one lymphocyte does not react to a multiplicity of antigens; and that antigen-induced proliferative reactions of this magnitude are restricted in unimmunized animals to the major histocompatibility isoantigens within the same species.

Materials and Methods

The isogenic rat strains Brown Norway (BN), Lewis (L), DA, and their various F_1 hybrids used as donors for the lymphocyte cultures have been described previously (14–15). These strains differ by major histocompatibility isoantigens determined by the AgB locus. Donors for some of these studies were tolerant animals generously supplied by Dr. W. K. Silvers. These had been rendered tolerant of the histocompatibility isoantigens of another strain by inoculation at birth with 50 million homologous bone marrow cells. Tolerance in these recipients was indicated by the permanent survival of test skin homografts (16, 17).

The preparation of peripheral blood leukocytes and the conditions under which they were cultured have also been previously described (4, 9).

Proliferation was assessed by the incorporation of tritiated thymidine (^{3}H-TdR)[2] into newly synthesized DNA and was measured quantitatively by liquid scintillation spectrometry. The same procedures as before were employed except for certain minor modifications (9). Protein carrier was omitted. The trichloroacetic acid (TCA)-precipitated pellets were washed one further time in 3 M TCA, then solubilized for 30 min. at room temperature in 2 M NH_4OH. 0.1 ml samples were transferred to disposable scintillation vials containing 2.5 ml of scintillation fluid and counted for 10 min. The results are expressed as mean counts per minute per culture.

For some of the experiments, the identity of the responding cells was determined by examination of chromosome spreads for sex chromosome markers (5, 9). This material was prepared according to procedures described by Moorhead et al. (18).

Where appropriate, additional specific details of the experimental protocol are presented with the Results.

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[2] ^{3}H-TdR: Methylthymidine-^{3}H, specific activity 6.7 c/mM; New England Nuclear Corp., Boston, Mass. 0.25 μc/0.1 ml was added to each culture.

RESULTS

Attempts to Demonstrate the Presence of "Recruitable" Cells among the Peripheral Blood Lymphocytes of Immunologically Tolerant Donors

One possible explanation for the large proportion of parental strain lymphocytes which can be stimulated by a major histocompatibility antigen system present on F_1 cells is that two distinctly different kinds of cellular response may be involved: (*a*) A small number of specifically responsive cells initially react to the antigen(s)—(These may represent antigen-sensitive cells (ASC's) which bear specific antigen receptor sites on their surfaces, and their response may or may not involve cell proliferation); and (*b*) A large number of other lymphocytes in the population are nonspecifically engaged or "recruited" as the result of some activity of the specific minority. Presumably, a large porportion of these recruitable cells observed in the response to one antigen could be induced equally well to respond to a second antigen system by a different subpopulation of specific cells.

Results obtained in a previous study provide some evidence against this possibility; namely, in mixed cultures of cells from parental strain and F_1 hybrid donors, the F_1 cells do not participate by entering the mitotic cycle themselves in significant numbers (4, 5). Nevertheless, there may be a requirement that the specific and the recruited cells be genetically more similar in order for some interaction to occur between them.

The possibility remains, therefore, that recruitable cells may exist among the lymphocyte population of an immunologically tolerant isologous animal. Although cells from tolerant donors do not respond in mixed cultures with F_1 hybrid cells bearing the tolerance-inducing antigens, this nonreactivity may reflect the inability of these cells to initiate a proliferative reaction. Presumably, this would stem from the deficit induced with the tolerant state in the small subpopulation of specific "triggering" cells. These missing specific cells might therefore be replaced in a mixed culture system simply by supplying a population of lymphocytes from a normal, isologous donor.

Accordingly, "3-way" mixed cultures were established consisting of parental strain lymphocytes from both tolerant and isologous normal donors and of cells from F_1 hybrid donors possessing the tolerance-inducing antigens. Normal and tolerant parental strain donors of different sexes were used so that the origin of the proliferating cells could be established by examining the sex chromosomes of the mitotic figures. Each 1 ml culture contained 3 million lymphocytes—1 million from each of the three types of donors. The cultures were terminated on the 4th and 5th days after 16 hr exposure to ^{3}H-TdR[2] or to 4 hr of colchicine.[3]

The results of a representative experiment are presented in Table I. In group A, cells from three different L males, tolerant of the isoantigens of the DA strain,

[3] Colchicine: 0.5 μg/ml final concentration.

were cultured with normal L female cells and with L/DA F_1 hybrid female cells. Of a total of 132 metaphase plates examined, only 5 were from the tolerant male donor. Group B cultures consisted of cells from the same three tolerant animals mixed with F_1 cells only (1 million plus 1 million). There was some background activity discernable among the cells from the tolerant donors; however, the incorporation of ^{3}H-TdR and the mitotic indices were much lower than in group A. This proliferative activity might be indicative of a less than complete degree

TABLE I

Attempts to Detect the Presence of "Recruitable" Cells among the Lymphocyte Population Derived from Tolerant Donors

Group	Combination	Day 4				Day 5			
		♂	♀	MI*	^{3}H-TdR‡	♂	♀	MI	^{3}H-TdR
				%	*cpm*			%	*cpm*
A	L ♂ No. 6 (tol DA) + L ♀ + L/DA	1	24	1.0	616	1	24	1.8	1377
	L ♂ No. 8 (tol DA) + L ♀ + L/DA	1	24	1.7	1005	0	12	1.8	1953
	L ♂ No. 34 (tol DA) + L ♀ + L/DA	2	23	0.6	1446	0	25	2.1	2905
Totals		4	71			1	61		
B	L ♂ No. 6 (tol DA) + L/DA	7	0	<0.1	202	12	0	8.0	480
	L ♂ No. 8 (tol DA) + L/DA	16	0	0.5	367	7	0	1.5	1087
	L ♂ No. 34 (tol DA) + L/DA	0	0	<0.1	132	4	0	<0.3	346
C	L ♀ + L/DA	0	18	1.8	1083	0	7	2.2	1710

* Mitotic index based on the number of mitotic figures per 500 cells.

‡ ^{3}H-TdR incorporation; mean of triplicate cultures. Individual values did not vary by more than 15% of the mean.

of tolerance. All chromosome spreads were examined "blind", and groups B and C were included primarily as controls for the validity of the sex chromosome scoring. The results indicate that recruitment of lymphocytes from a tolerant donor by the activities of cells from a normal isologous donor does not occur to any significant extent; if it did, one would expect that as many as 50% of the metaphase figures would be from the tolerant donor.

The Effect of Multiple Antigenic Differences on the Response

If the parental strain cells which respond in mixed cultures of lymphocytes from a particular combination of parental and F_1 donors are of limited potential

and can react to only one of several antigen systems, it follows that proliferative responses in cultures of parental cells exposed to multiple antigenic systems simultaneously should approximate the sum of the responses to the various antigen systems presented separately.

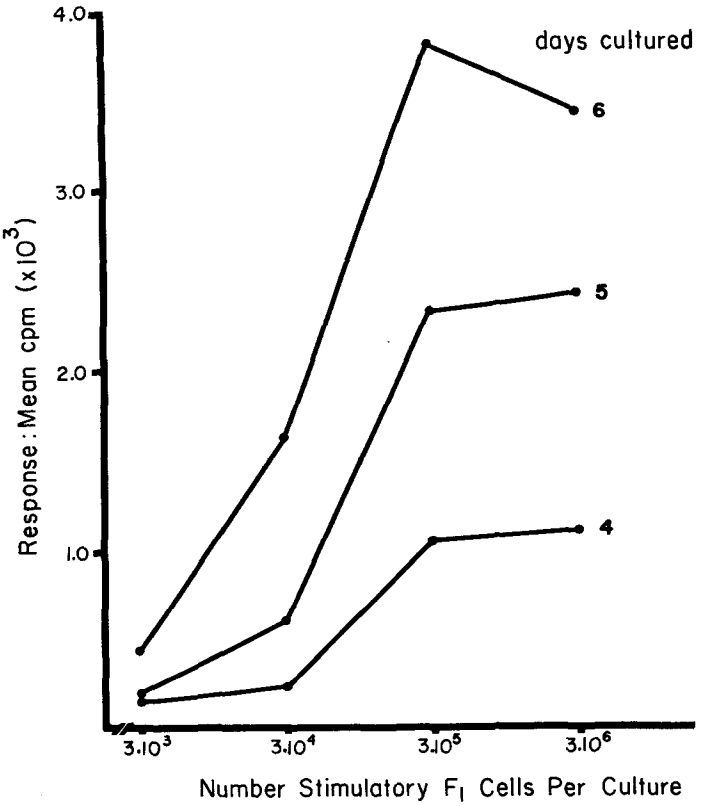

FIG. 1. Curves describing the dose-response relationship of proliferation in a MLI with constant number of parental strain lymphocytes exposed to increasing numbers of stimulating F_1 cells. 2.0 million BN strain lymphocytes were exposed to varying numbers of DA/BN F_1 cells and the proliferative response measured on days 4, 5, and 6.

This experiment requires that the responding parental cells be exposed to a "saturating" dose of F_1 antigen-bearing cells, i.e., that the proliferative response is not altered by the simple addition of more stimulatory cells bearing the same antigen. Accordingly, a dose-response curve for BN strain lymphocytes exposed to varying numbers of DA/BN F_1 cells is presented in Fig. 1. These cultures consisted of 2 million BN lymphocytes mixed with 3 thousand–3 million F_1 cells; the cultures were terminated on days 4, 5, and 6 after 16 hr exposure to ^{3}H-TdR. The data show that maximal responses were obtained with 300,000

stimulatory cells. Similar results were obtained with other strain combinations, and therefore, 500,000 cells was the dose of F_1 cells selected for use in the following experiments.

TABLE II

Proliferative Responses by Parental Strain Rat Lymphocytes Exposed to (a) Single or Multiple Antigen Systems and (b) Single or Multiple Genetic Determinants of a Given Antigen System

Group	Strain combination	AgB genotype	Response* ^{3}H-TdR incorporation		
			Day 3	Day 4	Day 5
			cpm	*cpm*	*cpm*
A	L + L/DA	1/1 + 1/4	441	1107	2564
	L + L/BN	1/1 + 1/3	430	1348	2934
	L + DA/BN (tol L)	1/1 + 4/3	898	2130	4420
	BN + L/BN	3/3 + 1/3		450	3113
	BN + DA/BN	3/3 + 4/3		1080	5056
	BN + L/DA (tol BN)	3/3 + 1/4		1421	7183
B	DA + BN/DA	4/4 + 3/4		5381	9137
	DA + BN (tol DA)	4/4 + 3/3		5498	10983
	DA + L/DA	4/4 + 1/4	1196	5503	7005
	DA + L (tol DA)	4/4 + 1/1	1525	5569	8325
	BN + L/BN	3/3 + 1/3	341	741	2285
	BN + L (tol BN)	3/3 + 1/1	249	698	2692
C	*controls, unmixed*:				
	L, DA, BN, L/DA,				
	L/BN, DA/BN				
	tol. parental strains		<50	<100	<200
	tol. F_1s				
	controls, mixed tol:				
	DA/BN (tol L) + L/DA (tol BN)				
	BN (tol DA) + BN/DA				
	L (tol DA) + L/DA		<50	<200	<300
	L (tol BN) +L/BN				

* Mean cpm of triplicate cultures; individual values did not vary from the mean by more than 15%.

To compare the effect of single and of multiple isoantigenic systems on responding parental strain cells in the MLI, various combinations of parental strain (A) lymphocytes were cultured with cells from F_1 hybrid donors (A/B), (A/C), or (B/C) (Table IIA). With the use of A/B or A/C cells, parental A

strain lymphocytes are thereby exposed to one or the other of two different strong isoantigen systems (B or C) separately, while cells from B/C donors provide both the different antigen systems simultaneously. In order that the proliferative response in the A + B/C cultures be unidirectional and involve only the A strain cells, as in the A + A/B and A + A/C cultures, the B/C donors were rendered tolerant of the A strain isoantigens by inoculation at birth with bone marrow cells from A strain donors [hence, B/C (tol A)].

The mixed cultures consisted of 1 million parental strain and 0.5 million F_1 hybrid cells in a total volume of 1 ml. Appropriate control cultures consisting of parental and F_1 cells cultured separately were included. Other controls employed cells from the tolerant donors cultured with F_1 cells bearing the tolerance-inducing antigens (Table IIC). ^{3}H-TdR was added to the cultures for a 16 hr period prior to terminating them on days 3, 4, and 5.

The results of one experiment with parental and F_1 cells derived from various donor combinations are presented in Table IIA and IIC, and are typical of results obtained in 13 independent experiments. Cultures of L lymphocytes exposed to both DA and BN antigens, or of BN cells exposed to both L and DA antigens simultaneously, show more proliferation than parallel cultures of these parental strain cells exposed to the same isoantigen systems singly.

A previous study showed that the magnitude of the proliferative response in the MLI depended on the particular strain combination of donors employed (4). The data in Table IIA extend this conclusion and show that reactivity of lymphocytes exposed to multiple antigen systems is approximately additive: (*a*) ^{3}H-TdR incorporation by parental cells in those donor strain combinations where the separate response to the two antigens are of *similar* magnitude (i.e., L + L/DA and L + L/BN) was nearly doubled when the antigens were presented simultaneously [L + DA/BN (tol L)]; and (*b*) with combinations in which the separate responses to the two antigens are markedly *dissimilar* (i.e., BN + L/BN and BN + BN/DA), the combined response [BN + L/DA (tol BN)] is nearly additive.

The Effect of the "Gene-Dosage" of an Isoantigenic System on the Response in the MLI

Since the magnitude of proliferative reactivity by parental cells in mixed cultures depends in part on the number of antigen systems present, it is important to determine what effect the "genetic dose" of a given isoantigen system might have on the proliferative response. Accordingly, parental strain lymphocytes were cultured with antigen-bearing cells from F_1 hybrid donors and from a homologous parental strain donor (Table IIB and IIC). The homologous donors were made tolerant of the isoantigens of the responding cell donors to insure that reactivity in the mixed cultures would be unidirectional. Control cultures, the same as the ones described above, were included.

The results, presented in Table IIB show that there is no difference in the response of 1 million DA strain lymphocytes to 0.5 million antigen-bearing cells whether they are derived from an F_1 or from a homologous donor.

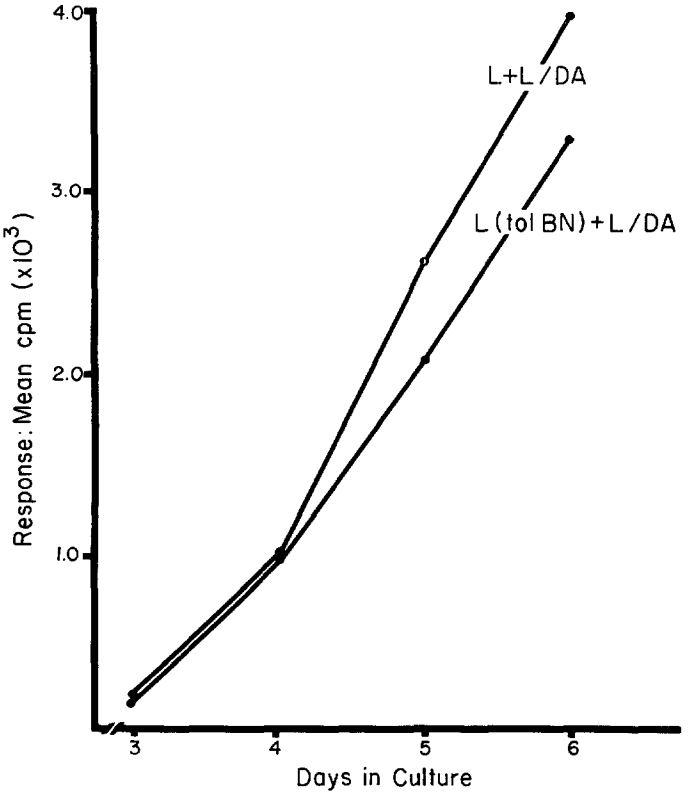

FIG. 2. Curves describing the proliferative activity of lymphocytes from a rat tolerant of one histocompatibility antigen system stimulated by a different antigen system. Normal L or L(tol BN) lymphocytes were cultured with L/DA cells. The responses were similar, indicating that tolerance to one antigen system does not alter the response capacity to a different antigen system.

Effect of a State of Induced Tolerance on the Proliferative Response to a Different Antigen System

Previous studies have demonstrated that the lymphocytes from tolerant animals in mixed cultures with F_1 hybrid cells bearing the tolerance-inducing antigens show either no, or markedly diminished, proliferative activity (5). This suggests that the fraction of parental cells which would have responded have been specifically destroyed or inactivated by some unknown process as a con-

sequence of the induction of tolerance. If the reactive cells in the MLI are limited in their response capacities, it follows that a state of induced tolerance to one isoantigen system should not affect the proliferative activity of the lymphocytes to a different antigen system. Preliminary data in a previous study suggested that this was the case (5).

A further test of this premise was made by comparing the proliferative activity of parental strain peripheral blood lymphocytes, derived from panels of normal L donors or from littermates made tolerant at birth to isoantigens of the BN strain, cultured with L/DA F_1 hybrid cells. Cultures were terminated on days 3–6, after 16 hr exposure to ^{3}H-TdR, as before. Typical results, shown in Fig. 2, indicate that in the MLI the induction of a state of immunological tolerance to one isoantigen system does not alter the response to a second antigen system to any significant extent.

Proliferative Activity of Rat Lymphocytes in a Homologous MLI and in a Heterologous MLI

If, as the preceding experiments suggest, the lymphocytes that are reactive to a particular histocompatibility system are unipotential, and at the same time, they consist of 1–3% of the total number of lymphocytes available (9), it follows that only a few antigen systems can stimulate proliferative responses of the magnitude seen with the MLI. One possibility is that these are limited to the major histocompatibility antigens of the species. It might therefore be predicted that the strong histocompatibility antigens capable of stimulating a large number of homologous lymphocytes of one species should not induce any significant proliferation of lymphocytes from a different species.

This reasoning was tested in a preliminary manner with the use of mixed cultures of lymphocytes derived from human and rat donors. Mixed cultures consisting of the following combinations (see Table III) were established:

(A) Normal lymphocytes from three different DA rats (No. 1–3) plus human cells pretreated with mitomycin C (DW-m).

(B) Lymphocytes from three different DA rats (4–6), previously immunized with human leukocytes, plus DW-m cells.

(C) DA No. 1–6 lymphocytes plus homologous DA/BN F_1 cells.

(D) Homologous human mix, MM or CN (without mitomycin C treatment) plus DW-m.

and, for control purposes:

(E) Parental strain-F_1 rat lymphocyte mixed cultures in the presence of DW-m cells (to insure that the latter are not toxic to the rat lymphocytes).

(F) Rat cells unmixed.

(G) Human cells unmixed.

The human lymphocytes were pretreated with mitomycin C to prevent any

TABLE III

Comparison of Heterologous and Homologous MLI

Group	Culture	Response* ^{3}H-TdR incorporation		
		Day 4	Day 5	Day 6
		cpm	*cpm*	*cpm*
A. Heterologous MLI: Normal rat lymphocytes and human lymphocytes treated with mitomycin C				
	DA (norm) No. 1 + DW-m	596	729	661
	2 +	460	389	356
	3 +	318	492	473
B. Heterologous MLI: Presensitized rat lymphocytes and human lymphocytes				
	DA (sens) No. 4 + DW-m	3221	6951	4150
	5 +	2078	4184	1981
	6 +	1170	1476	994
C. Homologous MLI: Parental and F_1 rat lymphocytes				
	DA No. 1 + DA/BN	1010	1701	2833
	2 +	1314	1999	2303
	3 +	1001	2814	1901
	4 +	1127	2640	3831
	5 +	1000	2419	2793
	6 +	789	1793	2747
D. Homologous MLI: Human lymphocytes				
	MM + DW-m	1046	1435	3323
	CN +	2078	3483	5706
E. Homologous–Heterologous MLI:				
	DA No. 1 + DA/BN + DW-m	1543	1603	2857
	2 + +	1484	1993	2415
	3 + +	1705	1251	2053
F. Control				
	DA No. 1–6, DA/BN	<100	<200	<200
G. Control				
	MM, DW-m, CN	<50	<100	<100

* Mean of triplicate cultures; individual values differed from mean by <15%.

DNA synthesis and cell division on their part.[4] The DA rats were immunized by a single injection of human leukocytes subcutaneously in the axillary regions (30 million leukocytes per site, 2 sites) 8 days prior to their use as cell donors.

[4] The cells were incubated at a concentration of 10 million per ml in media (4) containing mitomycin C 25 μg/ml for 20 min. at 37°C. They were then washed three times in media before use in cultures.

The results of this experiment are presented in Table III. They show that human cells do not stimulate more than barely detectable proliferation of rat lymphocytes unless the latter are derived from immunized donors. It appears that the circulating lymphocyte pool contains may more cells capable of recognizing and responding to antigens within the species than to comparable antigens of different species.

DISCUSSION

It seems clear that the proliferative behavior of lymphocytes in the MLI has some immunologic basis; however, its exact relationship to the cellular events in the immune response mechanism of the intact animal is not yet clear. On the basis of the results of this and several previous studies, we believe that stimulation of cell division in the MLI is the outcome of an immunologic recognition reaction triggered by histocompatibility antigen determinants which act upon specific receptor sites located on potentially responsive, immunologically competent lymphocytes.

Recent investigations with this culture system have suggested that of the circulating lymphocyte population in rats, approximately 1 cell in 50 is responsive to the complex of isoantigenic specificities associated with any one of a series of alleles present at a single major histocompatibility locus (9). In vivo, a similar number of homologous lymphocytes has been shown to initiate GVH reactions in mice and birds when introduced into an environment where they are exposed to a major transplantation antigen difference (10–13). This is at least three orders of magnitude in excess of the 10^{-5} frequency of antigen-sensitive cells (ASC's) responsive to heterologous erythrocyte antigens (19–21); therefore, it is difficult to reconcile this large number of active cells in the MLI with the clonal selection hypothesis, which predicts that the receptor sites possessed by a given antigen-sensitive lymphocyte would be of single or limited specificity. 50 of these antigen systems would theoretically exhaust the response capabilities of the total circulating lymphocyte population.

Of the several different explanations previously advanced to account for this dilemma (9), the data presented in this communication favor the following interpretation: that the reacting cells in the MLI are restricted in their response potentialities as the clonal selection theory would predict, but that there is a very limited number of antigen systems—perhaps 30—capable of stimulating as many as 2% of the cells, and that these are the major histocompatibility (H) isoantigens of the species.

Assuming that as many as half of the circulating lymphocytes of the adult rat, estimated to number 2×10^9 (22), are committed to react to the major species (H) antigens, this would leave 10^9 reactive to other antigens of the immunologic universe. Some of these cells—perhaps as many as half of them—undoubtedly represent expanded clones of ASC's, reflecting previous exposures to various environmental antigens. However, assuming the frequency of ASC's responsive to other antigens to be of the order of 10^{-5}–10^{-6} each in unimmunized animals, the remaining 5×10^8 lymphocytes would be available to react to a great many different determinants.

As has been pointed out before, the large number of responsive cells in the MLI

may represent the stimulation of numerous different ASC's, each present in a frequency of approximately 10^{-5}, by a large number of different antigenic specificities associated with the AgB histocompatibility locus (9). This possibility is unlikely since it would require that two isogenic strains of rats incompatible at the AgB locus differ by 1000 or more antigens determined by this major locus alone. These strong histocompatibility loci do constitute multiple alleleic systems, and even in the most characterized species, the mouse, the possible upper number of antigen specificities has not been determined, but some estimates place it at around 35 (23). Furthermore, this hypothesis provides no explanation for the greater immunologic responsiveness of a lymphocyte population against homologous, rather than heterologous, antigens.

The results of the above experiments also rule out the possibility that a significant number of the responding cells in the MLI are stimulated by some nonspecific process. Lymphocytes obtained from tolerant animals could not be recruited or coerced to proliferate in the presence of cells from normal, isologous donors which were responding to F_1 cells bearing the tolerance-inducing antigens.

Furthermore, the present data are not compatible with the possibility that a significant proportion of the lymphocytes which react to one H isoantigen system are responsive to others. The degree of proliferation in the MLI depends on the number of different antigens presented to the responding population, and not on the number of determinants or "gene dosage" of a given isoantigen. A state of induced immunologic tolerance to one antigen system does not quantitatively alter the response of cells from such a donor to a second isoantigen system. However, it must be pointed out that the possibility remains that of the 2% of the cellular population which are demonstrably reactive to one isoantigen system, 2% of these on a random basis, might be reactive to a second strong isoantigen system. These would not be detectable with the experimental design employed, since they would number about 1 cell in 2500, or approximately 0.04% of the total population.

In the past, it has been difficult to demonstrate additive proliferative responses with the use of two different (nontransplantation) antigens simultaneously and lymphocytes from individuals sensitized to both of them. In this case, part of the difficulty in demonstrating an additive response may stem from the use of nonsaturating concentrations of antigen in the culture. In support of the present concept, Albertini and Bach (24), using human donors, demonstrated greater magnitudes of response when mitomycin C-treated leukocytes possessing two different strong H isoantigens determined by two different alleles, rather than one, at the HL-A locus were employed as stimulatory cells.

Accepting, then, that the cells which react to a given isoantigen system are ASC's, unipotential in their response capacities, and that there is little, if any, nonspecific activation of these cells from a resting to a mitotically active status, then it follows that the number of antigen systems capable of provoking such

a large response, involving 2% of the lymphocyte population, must number considerably less than 50. The exact nature of these antigens is far from clear; however, it appears that they represent the strong H isoantigens of the species. This implies that (*a*) "strong" transplantation isoantigens possess that property by virtue of the fact that a large number of lymphocytes can recognize and respond to them, and conversely, "weak" antigens which constitute a negligible proliferative stimulus are those for which there are smaller numbers of reactive cells; and (*b*) lymphocytes from donors previously immunized with a weak antigen, and therefore possessing expanded clones of reactive cells, would demonstrate significant stimulation in cultures by such an antigen.

In support of this view, previous studies with rat donors of different strains demonstrated that in order to provoke measurable proliferative activity in the MLI the codonors must differ by isoantigens determined by the major AgB H locus (4, 6). Also, Dutton's studies indicate that murine splenic lymphocytes can be demonstrably stimulated with the use of donors differing by a single H specificity at the H-2 locus, but not with single differences involving the weaker, non–H-2 loci (2).

With respect to the effect of presensitization against major H isoantigens on the behavior of lymphocytes in the MLI, the data are in conflict. Some investigators have reported a diminished response on the part of lymphocytes from donors that have been previously immunized (5), while others find that the proliferative activity is augmented over that found with cells from normal donors (25). At this point, studies with donor combinations involving weak H isoantigen differences, and where one of the donors has been preimmunized to these antigens, are only fragmentary; further definitive studies are required, particularly with the use of donors at various times after immunization.

From the arguments presented above, it can be predicted that (*a*) the strongly mitogenic antigen systems of one species, which can stimulate proliferative activity in homologous lymphocyte cultures, would be far less effective in stimulating heterologous lymphocytes, and (*b*) these heterologous antigens should become effective if the lymphocyte donors have been immunized against them. The results of the preliminary experiments reported in the section concerning heterologous mixed cultures and the recent report by Lafferty and Jones (26) suggest that this reasoning is correct, but studies with other heterologous donor combinations are in order. Lafferty and Jones employed GVH reactions to assess the comparative immunocompetence of lymphocytes in homologous and heterologous environments. They showed that avian lymphocytes of one species would not provoke detectable GVH reactions in hosts of a different species unless the lymphocyte donors were previously immunized, while primary reactions were demonstrable in homologous hosts. Furthermore, they demonstrated that heterologous cultures of sheep and rabbit lymphocytes did not undergo as much proliferation as occurred with homologous mixed cultures involving these species.

The importance of the immunologic capacity to recognize and respond to

certain isoantigens within the species with greater facility, on the basis of the number of potentially reactive cells available, rather than to heterologous antigens of different species, is not clear. However, it could have some bearing on the biological significance and purpose of H isoantigens (27). The origin of the large number of cells that are reactive to these species of isoantigens is an important point that remains to be established. They could represent an expanded clone of "memory" cells remaining as a consequence of previous experiences with altered "self" antigens (e.g., associated with incipient neoplasia) or with cross-reacting environmental antigens. Their presence, on the other hand, may be determined genetically; i.e., they may reflect a modification of the immune response mechanism, acquired through natural selection, which endows the individual with the capacity to react very promptly to certain antigens. An example of the latter might be the ability to ward off infection by species-specific pathogens which may have incorporated bits of cytoplasm containing histocompatibility antigens of the previous host, or whose own antigens may mimic host histocompatibility isoantigens. These and other possibilities have been recently considered by Snell (27). Studies on the proliferative activity of lymphocytes derived from animals raised in an antigen and pathogen-free environment might provide important information on this point.

Along these lines, Burnet (28) has suggested that adaptive immunity arose early in vertebrate evolution as a protective mechanism against neoplastic transformation of somatic cells that are antigenically distinguishable. He suggests that a wide polymorphism of histocompatibility antigens coupled with a mechanism for the generation of diversified immune receptor patterns were developed for this purpose.

Jerne (29) has recently extended this reasoning and proposes that (*a*) the germ line contains structural genes which code for antibody specifically directed against histocompatibility determinants of the species, (*b*) mutations occur involving the variable portion cistrons (v), thereby coding for specificities other than H antigens, and (*c*) the survival of these mutant cells is favored by the suppression of nonmutant antecedents. The suppression of nonmutants takes the form of tolerance induced in ontogeny to self antigens. Since no one individual of a species possesses all the H antigens of his species, the v genes of his germ line must code for antibodies to all his species antigens in order to insure reactivity against those he does possess. Consequently, Jerne's hypothesis predicts that the portion of the germ line cells with v genes determining antibodies against self antigens are suppressed and the survival of mutants of this germ line is thereby favored. On the other hand, cells expressing the other set of v genes which code for H antigens of the species that the individual happens not to possess are not suppressed, and are therefore available in large numbers for reactivity against H antigens of other members of the species.

SUMMARY

Studies were designed to provide some explanation for the unexpectedly large proportion (2%) of parental rat strain peripheral blood lymphocytes that are

reactive in the mixed lymphocyte interaction (MLI) to a strong homologous transplantation isoantigen(s) present on cells from an F_1 donor. The possibilities considered involve nonspecific activation and multispecific reactivity on the part of the responding cells.

The essential findings of this study were:

(*a*) In 3-way mixed cultures, lymphocytes obtained from tolerant animals were not "recruited" to proliferate in the presence of cells from normal, isologous donors which were in the process of responding to F_1 cells bearing the tolerance-inducing antigens. With the use of chromosome markers and normal and tolerant parental strain donors of different sexes, the responsive cells were identified and proved to be derived from the normal and not the tolerant donor.

(*b*) The magnitude of the proliferative response is increased additively when potentially reactive cells are exposed to two antigen systems simultaneously. On the other hand, doubling the "gene-dosage" of the genetic determinants of the H isoantigens employed had no effect on the responding cells.

(*c*) A state of induced immunologic tolerance to one H isoantigen system did not alter the response capacity of cells from such a donor to an alternative antigen system.

(*d*) Mixed cultures of heterologous cells from human and rat donors displayed a proliferative response which was less than that of homologous mixed cultures from human or rat donors. Prior sensitization of rat donors with human cells, however, greatly increased the mitotic activity of rat lymphocytes stimulated with human cells.

These results suggest that the large number of responsive cells in the MLI do not include a significant number recruited or activated in some nonspecific manner. Rather, they appear to be fully specific in their response capacities so that a given lymphocyte does not react to a multiplicity of different antigens. The degree of proliferation depends on the number of different antigen systems presented to the responding population and not on the number of genetic determinants or "gene dosage" of a given isoantigen system. Finally, on a cell-for-cell basis, the peripheral blood lymphocyte population contains more cells reactive to histocompatibility isoantigens within the species than to heterologous antigens of a different species.

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