QUANTITATIVE STUDIES ON THE MIXED LYMPHOCYTE INTERACTION IN RATS

VI. Reactivity of Lymphocytes from Conventional and Germfree Rats to Allogeneic and Xenogeneic Cell Surface Antigens*

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The proportion of lymphocytes present in an individual which are reactive to the major histocompatibility (H)[1] alloantigens of another member of the same species (histocompatibility antigen-reactive cells [H-ARC]) has been shown to be unusually large. Estimates derived from studies with the graft-*versus*-host (GVH) reaction in vivo (1–4) and with the mixed lymphocyte interaction (MLI) in vitro (5, 6) are in substantial agreement, and place this number at approximately 2–4% of the cells of the circulating lymphocyte pool. This conclusion is difficult to accept in view of the constraints of the clonal selection hypothesis and consequently, various alternatives of a general nature have been proposed to account for these findings (7, 8). These include the possibilities that the large proportion of H-ARC (*a*) involve a large number which are "recruited" or nonspecifically activated; (*b*) are multipotential, having the capacity to react to any one of a series of different antigen determinants including other H alloantigens; (*c*) represent the cumulative activity of a great many subpopulations of antigen-reactive lymphocytes, each present in low frequency and stimulated to proliferate by a very large number of antigenic differences which might exist between two individuals; (*d*) reflect the existence of a prior state of immunization in the cell donors to cross-reactive dietary and environmental antigens, or to altered surface specificities on somatic cells such as tumor-specific antigens on spontaneously arising and successfully suppressed neoplasms; (*e*) have a low stimulation threshold and are triggered to respond as a consequence of multivalent binding to low affinity receptor sites by antigenic determinants represented in high density on cellular membranes; or (*f*) represent a pool of antigen-reactive cells important in some not yet defined crucial step(s) of ontogeny and their high frequency is brought about by some unknown expansion mechanism under innate genetic control (i.e., Jerne's recent model [9]).

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[1] *Abbreviations used in this paper:* ARC, antigen-reactive cells; B, bone marrow-derived; con, conventional; gf, germfree; GVH, graft-*versus*-host; H, histocompatibility; H-ARC, histocompatibility antigen-reactive cells; m, mitomycin C-treated; MLI, mixed lymphocyte interaction; PHA, phytohemagglutinin; T, thymus-derived; TdR-^{3}H, tritiated thymidine.

The results of previous studies rule the recruitment-nonspecific activation possibility on two counts: (*a*) in mixed cultures of leukocytes from parental strain and hybrid rats of different sexes, very few or none of the dividing cells possessed the sex chromosome markers of the hybrid donor (10, 11), and (*b*) in three-way cultures involving lymphocytes from normal donors, tolerant isologous donors of the opposite sex, and F_1 animals, the responding cells were all identified as being derived from the normal parental strain donor (12). In this circumstance, cells from the immunologically tolerant donor could not be coerced to proliferate in an environment where lymphocytes from normal donors were reacting to the presence of F_1 cells possessing the tolerance-inducing alloantigens.

Strong, but not conclusive evidence against the possible multipotentiality of H-ARC has also been provided by the finding that: (*a*) the magnitude of the proliferative response in the MLI is increased additively when lymphocyte populations are exposed to two H alloantigen systems simultaneously (12); (*b*) a state of immunologic tolerance to one H antigen system does not alter the response capacity of cells from such a donor to an alternative antigen system (12); (*c*) lymphocytes from previously immunized donors display a more prompt proliferative response against F_1 cells bearing these immunizing antigens than against others (6); and (*d*) populations of lymphocytes stimulated with one H alloantigen system, then exposed to BUdR and light energy (13) or to tritiated thymidine of high specific activity (14) to destroy the responding cells, nevertheless retain the capacity to respond to unrelated antigens including other H alloantigens. These findings are best interpreted with a model in which separate subpopulations of thymus-derived (T) cells react to different antigenic determinants.

The possibility that the high frequency of H-ARC represents the activity of a large number of subpopulations of antigen-reactive cells stimulated by an equally large number of antigens in the MLI lacks any supportive evidence. This premise would require 3000 different antigens to stimulate 3000 different subpopulations of antigen-reactive cells each present in a frequency of 10^{-5}. Individuals possessing different alleles of the major *H* locus, the one responsible for stimulation in the MLI, can be shown to differ at most by only 15 antigenic determinants.

Finally, the findings that lymphocytes do not produce substantial GVH reactions in xenogeneic hosts and are not stimulated to the same degree in the interspecies MLI as in the intraspecies MLI is difficult to reconcile with the low stimulation threshold-multivalent binding explanation (12, 15).

The present studies involved the use of lymphocytes from conventional and germfree rats in the allogeneic and xenogeneic MLI and were designed to test the possibility that the large number of H-ARC reflects the existence of a prior state of sensitivity to ubiquitous environmental and dietary antigens. The results demonstrate that circulating lymphocytes from germfree animals are fully reactive to allogeneic H alloantigens and display no demonstrable reactivity to surface antigens on xenogeneic cells. In contrast, lymphocytes from conventional rats display a reduced but definite level of reactivity to xenogeneic cells.

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Materials and Methods

Cell Suspensions.—The procedures used to obtain leukocytic and splenic cell suspensions have been described elsewhere (5, 6, 10–12). Peripheral blood was collected into citrate anti-coagulant by caridac puncture from rats and guinea pigs and by venipuncture from humans. Leukocytes were prepared by dextran sedimentation of whole blood; splenic cells were obtained from mouse and hamster spleens by passing minced fragments through a 50-gauge mesh sieve.

When necessary, cell suspensions were pretreated with mitomycin C (25 μg/ml, 10 million cells/ml for 25 min at 37°C, occasional agitation) after they were washed twice with Hanks' balanced salt solution. This procedure was used to obtain cells which could stimulate untreated lymphocytes while not synthesizing DNA themselves. During the course of these studies, it became clear that in rat-rat cultures, mitomycin C-treated F_1 cells were somewhat toxic to the potentially responsive nontreated parental lymphocyte population, although not to cells of other species. This shortcoming was partially overcome by using mitomycin C stock solutions that were filtered (0.22 μ Millipore VC filters [Millipore Corporation, Bedford, Mass.]) and washing the treated cells three times and including two 10-min incubation periods at 37°C in the wash procedure.

Cultures.—The procedures used for establishing mixed lymphocyte cultures have been described extensively in earlier publications (5, 6, 10–12). Mixed allogeneic lymphocyte cultures were initiated with cell suspensions from two different strains of rat donors; one of the suspensions was pretreated with mitomycin C. Xenogeneic mixed lymphocyte cultures consisted of untreated rat lymphocytes plus mitomycin C-treated human, hamster, mouse, or guinea pig lymphocytes. Unless stated to the contrary, all cultures were conducted in triplicate, and each culture consisted of 2 million cells (1 million from each of the donor suspensions) in Eagle's minimal essential medium supplemented with 5% freshly prepared BN rat serum. Unmixed cultures contained 2 million cells.

At various times, tritiated thymidine (TdR-^{3}H) (0.25 μCi in 0.05 ml medium; specific activity 6.7 Ci/mM; New England Nuclear Corp., Boston, Mass.) was added to the cultures; they were then harvested 16 hr later, treated with trichloroacetic acid (TCA), and the activity assessed with scintillation spectrometry (5).

Animals.—The animals employed in these studies consisted of rats of the isogenic Brown Norway (BN), DA, Lewis (L), Black Hooded (BH), and Fischer (F) strains which differ with respect to major H alloantigens determined by the AgB locus (16, 17); mice of the C57BL strain; hamsters of the MHA strain; Strain 2 guinea pigs; and three human donors (CN, MM, and DW). When cells from these donors were treated with mitomycin C this is designated (m).

Germfree (gf) rats consisted of sublines of the Fischer (F-344) strain maintained at the Lobund Laboratories, Notre Dame University, South Bend, Ind., and also were supplied by A. R. Schmidt (Grand Island Biological Co.), Madison, Wis., and Charles River Breeding Laboratories, Inc., Wilmington, Mass. The activity of cell suspensions from (gf) rats in mixed cultures was compared to that of conventional aimals (con) of the same age from the same colonies maintained at these locations.

Immunizations.—This was accomplished by subcutaneous inoculation of 30–40 million leukocytes or splenic cells bilaterally in the axillary regions. The animals were used as cell donors for the MLI 6–8 days later (6).

RESULTS

Preliminary Considerations: Toxicity and Dose Studies of Mitomycin C-Treated Allogeneic and Xenogeneic Stimulatory Cells.—In a previous publication, we

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presented preliminary evidence that lymphocytes from nonimmunized rat donors were markedly less reactive to the cell surface antigens of mitomycin C-treated xenogeneic cells than to the surface alloantigens on leukocytes from F_1 rat donors (12). Table I is an example of the results of one such experiment. These findings were interpreted as an indication that the frequency of cells in the circulating lymphocyte pool of an individual reactive to alloantigens of other members of the same species is much higher than the frequency

TABLE I

Stimulation of Rat Lymphocytes with Cells from F_1 Donors in the Allogeneic MLI and from Human Donors in the Xenogeneic MLI

Culture	Response* Mean cpm/culture		
	Day 5	Day 6	Day 7
Allogeneic MLI			
BN + BN/DA	1896	3782	2819
CN + DW(m)‡	5527	7968	5921
Xenogeneic MLI			
BN(norm) + DW(m)	976	5000	207
BN(sens)§ + DW(m)	1804	2744	2317
Controls			
BN(norm)	212	146	88
BN(sens)	198	138	89
BN/DA	108	92	77
CN	160	592	282
DW(m)	77	65	71

* Mean of triplicate cultures; individual variation from the mean did not exceed 15%.

‡ Cells pretreated with mitomycin C.

§ Animals sensitized subcutaneously 7 days earlier with 30 million DW human leukocytes.

reactive to the cell surface antigens of other species. Other possible explanations for the difference in the responsiveness of rat cells in the allogeneic and xenogeneic MLI are: (*a*) mitomycin C-treated cells may be more toxic to the responding rat cells than untreated cells are; (*b*) human cells may be more toxic to rat cells than allogeneic cells of F_1 origin; or (*c*) human leukocytes may possess fewer surface antigens than rat cells do and that comparable degrees of stimulation with xenogeneic cells requires the presence of greater numbers of them.

These explanations all seemed unlikely in view of the previously reported findings that mitomycin C-treated human cells do effectively stimulate lymphocytes from a different human donor and also cells from rats previously immunized with human leukocytes. However, on further examination of these possibilities, it was observed that mitomycin C-treated rat cells did partially

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inhibit the reactivity of untreated potentially responsive rat cells, however, mitomycin C-treated human cells did not.

The possible toxicity of mitomycin C-treated cells was tested by comparing the response of parental strain rat lymphocytes to mitomycin C-treated leukocytes or to untreated leukocytes from F_1 hybrid donors (F_1 (m) or F_1). Aliquots of F_1 leukocytes were incubated with or without mitomycin C, washed, counted, and mixed with parental strain leukocytes as outlined above. Table II presents the results of nine experiments which indicate clearly that parental strain leukocytes were less reactive to F_1 cells which had been treated with mitomycin C. With the use of fewer F_1 (m) cells in the MLI, and including the precaution of two 10-min incubation periods at 37°C in the wash procedure, the apparent toxicity of F_1 (m) cells was reduced, but not eliminated.

TABLE II

Response of Parental Strain Lymphocytes Stimulated with F_1 Leukocytes Pretreated with or without Mitomycin C

Group*	Cultures	No. F_1 cells ($\times 10^6$)	Response,‡ mean cpm/culture					
			Day 4		Day 5		Day 6	
			Treated (m)	Non-treated	Treated (m)	Non-treated	Treated (m)	Non-treated
A	DA + L/DA	1.0	712	1512	920	2200		
	DA + BN/DA	1.0	88	3399	82	3889		
	DA + BH/DA	1.0			818	1659		
B	BH + DA/BH	0.5	1726	3184	2644	5668	1419	1710
	DA + L/DA	0.5	1375	2148	901	2479	1019	2889
	DA + L/DA	0.5	747	1466	677	2079	626	1764
C	F + F/BN	0.5	83	100	594	764	849	934
	F + F/BN	0.5	420	474	723	730	709	600
	DA + L/DA	1.0	731	1200	1288	2030	1217	2059
		0.3	827	1107	1496	1880	1105	2034
		0.1	448	598	1265	1277	1412	1785

* Group A cultures: 1.0 million F_1 or F_1(m) cells/culture; group B cultures: 0.5 million F_1 or F_1(m) cells/culture; group C cultures: fewer F_1(m) cells/culture, and F_1(m) cells subjected to 2–10-min incubations during washing.

‡ Mean of triplicate cultures; individual variation from the mean did not exceed 15%.

The possibility that human leukocytes are more toxic to potentially responsive rat cells than allogeneic cells are in the MLI was examined by determining what effect the addition of mitomycin C-treated F_1 cells or mitomycin C-treated human cells might have on the course of a MLI involving parental and F_1 (untreated) cells. 0.5 million mitomycin C-treated human or F_1 rat leukocytes were added to mixed cultures consisting of 1 million parental strain and 1

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million F_1 lymphocytes. Table III shows that addition of mitomycin C-treated xenogeneic cells generally brings about a slight increase in TdR-^{3}H incorpora-

TABLE III

The Effects of Including Mitomycin C-Treated Allogeneic $F_1(m)$ or Xenogeneic (Human) Leukocytes on the MLI

Culture	Response,* mean cpm/culture								
	Day 4			Day 5			Day 6		
	+ 0	+ F_1 (m)	+ DW (m)	+ 0	+ F_1 (m)	+ DW (m)	+ 0	+ F_1 (m)	+ DW (m)
DA + BN/DA	1127	803	1343	1640	1001	1603	1833	1544	2044
	1000	844	1484	1419	874	1093	2099	1320	2415
	789	478	705	793	503	843	1747	1284	2053
				1659	1263	1962	1844	1204	2077

* Mean of triplicate cultures; individual variation from the mean did not exceed 15%.

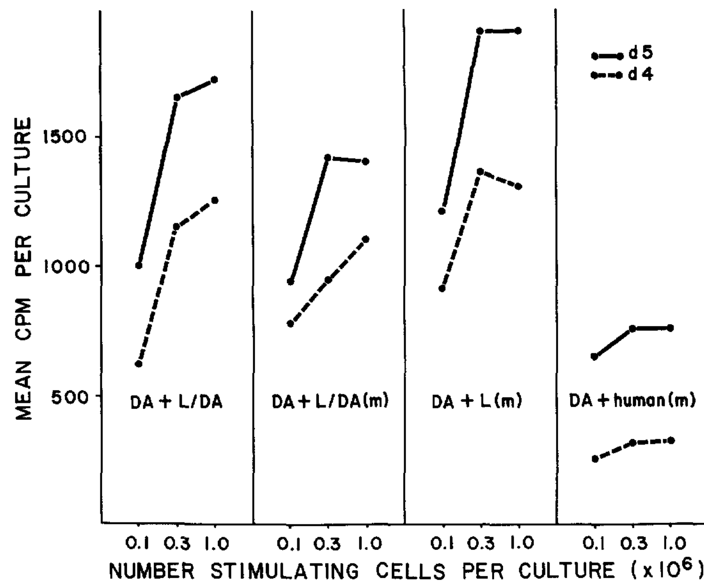

FIG. 1. The proliferative response of DA strain rat peripheral blood lymphocytes in the mixed lymphocyte interaction stimulated with various numbers of allogeneic or xengeneic cells.

tion during the course of a parental-F_1 MLI, whereas addition of F_1 (m) cells is somewhat inhibitory.

Fig. 1 presents data which show that the dose response relationships, i.e. varying the number of stimulatory cells, in the allogeneic and xenogeneic MLI are quite different. In this experiment, 1.5 million DA strain lymphocytes

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were incubated with 0.1 million, 0.3 million, or 1.0 million allogeneic L/DA, L/DA(m), L(m), or xenogeneic DW(m) or CN(m) leukocytes. The results show (*a*) F_1(m) cells are somewhat less mitogenic than are untreated F_1 cells; (*b*) mitomycin C-treated cells from parental donors are more mitogenic than F_1(m) cells; (*c*) xenogeneic cells are the least mitogenic; and (*d*) increase the dose of stimulatory cells beyond 0.3 million/culture of 1.5 million responding cells does not increase the magnitude of the response.

Over the course of several similar experiments, it was a consistent finding that mitomycin C-treated parental strain leukocytes were noticeably more mitogenic than comparably treated cells from F_1 donors. This was an unexpected finding in view of the results of previous studies that leukocytes from A/B F_1 donors and from B donors tolerant of A strain H isoantigens stimulated A strain leukocytes to a similar extent.

It can be concluded from these preliminary experiments that (*a*) xenogeneic (human) cells per se are not more toxic to potentially responsive rat lymphocytes than allogeneic F_1 cells are; but (*b*) they are less mitogenic to rat lymphocytes than comparably treated allogeneic F_1(m) leukocytes; and (*c*) the dose of stimulatory cells resulting in the maximum degree of proliferation of rat cells in the MLI is similar whether they are of allogeneic or of xenogeneic origin, however, the level of stimulation achieved is different.

These conclusions provide no support for the possibility that the comparatively smaller magnitude of proliferative responses by rat cells in the xenogeneic MLI is due to innate toxicity or to lack of mitogenicity (antigenicity?) of human cells. Rather a more likely possibility is that the subpopulation of circulating lymphocytes in the rat reactive to human cell surface antigens is considerably smaller than that reactive to allogeneic isoantigens.

Reactivity of Cells of Normal and Previously Immunized Rat Donors to Cells of Various Xenogeneic Species.—The finding that the magnitude of the proliferative response of rat lymphocytes stimulated in the xenogeneic MLI with human leukocytes is less than in the allogeneic MLI implies that on a cell-for-cell basis a larger proportion of the circulating lymphocytes of an individual are reactive to the histocompatibility alloantigens of other members of the same species than to comparable cell surface antigens of other species.

The results presented in Table IV provide further evidence which sustains this premise by showing that cells from various different species, hamsters, guinea pigs, mice, as well as human cells, are less stimulatory to rat lymphocytes than are cells from allogeneic F_1 donors. Furthermore, the response to xenogeneic cellular antigens in the MLI can be markedly increased if the rat donors have been previously immunized.

The data in Table IV were derived from 1 experiment and are fully typical of the results of 12 experiments with rat and human cells, 4 with rat and mouse, 3 with rat and hamster, and 2 with rat and guinea pig cells. In this experiment, pairs of DA rats were immunized with human or guinea pig leukocytes, or

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with hamster or mouse splenic cells. 7 days later, peripheral blood leukocyte suspensions were prepared from each of the rats and 1 million lymphocytes placed into culture with 0.5 million stimulating cells from L/DA rats or from

TABLE IV

Proliferative Response of Lymphocytes from Normal or Previously Immunized Rats to Allogeneic and Xenogeneic Cell Surface Antigens

Species combination	Culture combination		Results,* TdR-^{3}H incorporation cpm/culture		
			Day 4	Day 5	Day 6
Rat–rat	DA No. 1 (normal)	+ L/DA(m)	1466	2479	1764
	2	+	1202	1841	2213
Rat–human	DA No. 1 (normal)	+ DW(m)	420	481	317
	2	+	549	704	722
	3 (sens-human)	+	3646	4019	1604
	4	+	2901	3111	1299
Rat–hamster	DA No. 1 (normal)	+ MHA(m)	354	600	780
	2	+	498	722	542
	5 (sens-hamster)	+	2375	1704	1232
	6	+	1401	2984	1708
Rat–guinea pig	DA No. 1 (normal)	+ Strain 2(m)	236	498	304
	2	+	501	621	522
	7 (sens-guinea pig)	+	1522	1823	1703
	8	+	1201	2000	1309
Rat–mouse	DA No. 1 (normal)	+ C57BL/6(m)	218	499	569
	2	+	263	690	792
	3 (sens-human)	+	250	504	703
	4	+	272	412	517
	9 (sens-mouse)	+	904	870	1022
	10	+	1283	1122	1400
Human–human	CN	+ DW(m)	887	3489	6244
	MM	+	1244	2912	5819
	Controls: all unmixed cultures		<100	<200	<250

* Mean of triplicate cultures; individual variation did not exceed 15%.

one of the other species. The stimulatory cells were all pretreated with mitomycin C.

The results show the following: (*a*) Lymphocytes from normal rats are markedly less responsive to human, hamster, guinea pig, and mouse cells than to allogeneic cells. (*b*) Lymphocytes from previously immunized rats are more

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TABLE V

Comparison of the Proliferative Response of Lymphocytes from Germfree and Conventional Fischer Strain Rats in the Allogeneic and Xenogeneic MLI

Cultures	Response,* TdR-^{3}H incorporation cpm/culture											
	Exp 734			Exp 742			Exp 757			Exp 759		
	Day 3	Day 4	Day 5	Day 4	Day 5	Day 6	Day 4	Day 5	Day 6	Day 4	Day 5	Day 6
Allogeneic MLI												
F(con) + F/BN	54	659	1598	474	730	548						
+ F/BN(m)				420	723	709						
+ BN(m)							494	586	505			
+ F/DA	49	662	1684									
+ DA(m)							476	514	412	625	1910	1815
+ O	50	50	53				59	58	76	34	79	88
+ PHA	14,980											
F(gf) + F/BN				120	764	937						
+ F/BN(m)				83	594	849						
+ BN(m)								384				
+ F/DA	66	230	1109									
+ DA(m)								300		598	1780	1892
+ O	50	48	41					98		35	90	85
+ PHA	10,662											
Xenogeneic MLI												
F(con) + DW(m)				379	777	1457	118	246	233	198	789	812
+ C57BL/6(m)							94	162	193			
+ MHA(m)							238	414	732			
+ Strain 2 (m)							80	113	207			
F(gf) + DW(m)				75	114	97		66		88	102	99
+ C57BL/6(m)								57				
+ MHA(m)								85				
+ Strain 2(m)								71				
Controls (mixed)												
DA(m) + BN(m)							47	28	90			
DW(m) + DA(m)								65	33			
DW(m) + F/BN(m)				130	114	97						
MHA(m) + C57BL/6(m)								81	67			
CN + DW(m)				206	2429	3257	273	693	1574			
Controls (unmixed)												
CN				137	502	750	45	77	156			
Others								<50	<50			

* Mean of triplicate cultures; individual variation did not exceed 10–12% of the mean.

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reactive in the xenogeneic MLI when stimulated with cells from these various species. (*c*) The greater magnitude of proliferation of sensitized cells in the MLI is immunologically specific; lymphocytes from rats immunized with human cells show an increased response to human cells but not to mouse cells.

Reactivity of Lymphocytes from Germfree and from Conventional Fischer Strain Rats in the Allogenic and Xenogeneic MLI.— Table V is a compilation of the results of four different experiments which compare the proliferative response of lymphocytes from germfree and conventional Fischer 344 rats stimulated in the MLI with cells from allogeneic or from xenogeneic donors. Rats from sublines maintained at three different locations (see Materials and Methods) were used in these experiments.

For the first three experiments, cardiac blood was drawn into citrate anticoagulant, packed in wet ice, and flown to Philadelphia where the cultures were prepared. In the last experiment, the rats were shipped to Philadelphia in sterile "isolet" containers where blood was drawn from them immediately upon arrival and the cell suspensions prepared.

As before, all cultures contained 1.0 million responding cells and 0.5 million stimulatory cells, and the cultures were conducted in triplicate. The simulatory cells were derived from F_1 rats and other animals maintained at the University of Pennsylvania. The Fischer parents for the F_1 rat stocks were derived from the colony at Lobund Laboratories for the purpose of these experiments.

The results show that (*a*) lymphocytes from conventionally maintained Fischer donors were somewhat reactive to xenogeneic cells and were more reactive to allogeneic cells and (*b*) lymphocytes from germfree rats displayed no demonstrable reactivity to xenogeneic cells but were quantitatively normal in their response to allogeneic cells. There was no difference (one experiment) in the response of lymphocytes from (gf) or (con) animals to phytohemagglutinin (PHA-M, 0.01 ml/ml culture).

DISCUSSION

These studies show (*a*) that lymphocytes from conventionally maintained rats are less reactive to human, hamster, guinea pig, and mouse cell surface antigens than to the major H antigens of allogeneic cells, and (*b*) that lymphocytes from (gf) rats display no demonstrable reactivity to xenogeneic cells, but are quantitatively normal in their response to allogeneic cells. These findings confirm and extend the observations of Lafferty and Jones with cultures of sheep and rabbit cells (15), of Elkins with cells from (gf) rats in the GVH reaction (18), and our own previous studies with rat and human cells (12). The interpretation placed on these findings is that the circulating lymphocyte pool of an individual consists of a greater proportion of cells reactive to H alloantigens of other members of the same species than to the xenogeneic cellular antigens of members of other species.

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Along these lines, and accepting that the large number of cells reactive to H alloantigens (H-ARC) in the GVH and MLI are neither multipotential nor nonspecifically activated, it has been argued previously (*a*) that the number of different antigens capable of provoking responses of this magnitude must be limited, (*b*) that these are probably the "strong" H alloantigens of the species, and (*c*) that the biological distinction between strong and weak alloantigens may be on the basis of the frequency of antigen-reactive cells (ARC) capable of recognizing and responding to them.

These arguments predict that the strongly mitogenic "special" antigenic systems of a given species which can provoke proliferative responses involving large numbers of allogeneic lymphocytes should be ineffective in stimulating xenogeneic lymphocyte populations in the MLI unless the lymphocyte donors have been previously immunized to them. An individual of a given species may have a lymphocyte pool of sufficient size so that significant numbers of lymphocytes can be committed to reactivity to the various strong H alloantigen systems of his species, but not for comparable cell surface antigens of other species.

The response of lymphocytes from conventional animals to xenogeneic antigens described here and by Lafferty and Jones partially fulfills this prediction. However, even some proliferative activity to xenogeneic antigens is cause for concern because it indicates that a sizeable proportion of the lymphocyte population is reactive to these antigens. In this context, the nonreactivity of lymphocytes from germfree animals to xenogeneic antigens provides good evidence for the contention that the proliferative reactivity of cells from conventional animals to xenogeneic antigens reflects the existence of a prior state of immunization to cross-reactive environmental antigens. Furthermore, the lack of response to xenogeneic antigens, in spite of a normal response capacity to allogeneic antigens, suggests that the different subpopulations of lymphocytes reactive to allogeneic and to xenogeneic antigens in conventional animals arise by distinctly different mechanisms.

Concerning the possible mechanisms whereby the large number of lymphocytes reactive to H alloantigens are generated, the present findings with cells from germfree animals do not rule out the possibility that they stem from prior immunization as a consequence of exposure either to external dietary antigens or to internal somatic cellular antigens which might be variants of self antigens on spontaneously arising tumor cells suppressed by an immunologically-mediated surveillance mechanism.

Both of these possibilities, however, seem unlikely. It would be difficult to imagine how dietary antigens would be restricted in their cross-reactivity only to alloantigens to the exclusion of xenoantigens. Also, the possibility that prior immunization results as a consequence of the operation of a surveillance mechanism against variants of self antigens which cross-react with allogeneic

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antigens, presents a formidable conceptual difficulty. Given (*a*) a certain frequency of transformation of normal somatic cells to a neoplastic status, which probably involves genetic changes that are inheritable from cell generation to cell generation, and (*b*) that the appearance of altered antigens on the surfaces of somatic cells also involves genetic changes possibly at *H* loci genes, it is unlikely that these two mutational changes would occur together in the same cell at the same time to result in a neoplastic cell bearing immunologically recognizable surface antigens unless the neoplastic and surface antigen changes involve the same genetic event. As Burnet has pointed out (19), surveillance against neoplastic cells based on the operation of an immune response mechanism would in all probability require that neoplastic transformation of a somatic cell involve a cell which already expressed a mutationally altered self-antigen variant. This would imply that most normal nonneoplastic cells already possess antigenic variants in their cell surfaces which in turn predicts that *H* loci are unstable and mutate in high frequency. This prediction has not been sustained by immunogenetic studies of the behavior of histocompatibility alloantigens. Hence, at the present time there is no evidence that the strong H alloantigens and the large number of lymphocytes reactive to them are involved in any kind of immunologically-mediated surveillance mechanism.

Furthermore, the results of the present studies argue against another general possibility offered independently by several investigators to account for the large number of H-ARC, namely that a large number of cells having low affinity receptors for alloantigens are triggered to respond as a consequence of multivalent binding promoted by the high density of H determinants on the allogeneic-stimulating cell. Möller (20) has a recently described a model which suggests that the threshold of stimulation of thymus-derived (T) lymphocytes by antigen is lower than that for nonthymic, bone marrow-derived (B) lymphocytes, and that the high density of antigens on the surface membranes of allogeneic cells would allow for multivalent binding and the stimulation of large numbers of T lymphocytes having low affinities for the antigens involved. While this model has much merit and helps to explain some of the differences in behavior which B and T cells display upon administration of antigen, it does not adequately explain the large number of cells reactive to allogeneic H alloantigens. If a high frequency of lymphocytes from human A are reactive to alloantigens from donor B, and multivalent binding to cells having low affinity receptors and a low threshold of stimulation is the correct model to explain this phenomenon, then human cells and leukocytes from any other species should also be mitogenic for rat cells. The present studies do not confirm this prediction.

Thus the various possibilities which have been advanced to explain the large numbers of cells of the circulating lymphocyte pool reactive to allogeneic cell surface isoantigens have various shortcomings which have been discussed

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here and elsewhere (7, 8). Taken together, the information concerning the interaction of lymphocytes and alloantigens in the GVH reaction and in the MLI suggest that these represent a pool of unipotential ARC which are important by virtue of their functional capacity to respond to antigens of the species. The recent discovery (see ref. 21) that the immunologic capacity to respond to certain substances having a limited range of antigenic determinants is associated with various alleles of the major *H* loci in mice and guinea pigs suggests that this class of lymphocytes is involved in some crucial step(s) in the ontogeny of the immune response mechanism. Such a model has been recently proposed by Jerne (9), but whether this model is any better than the others remains to be tested by expert attention.

SUMMARY

The proliferative reactivity of lymphocytes from rat donors maintained under germfree or conventional conditions was examined in mixed lymphocyte cultures stimulated with allogeneic and xenogeneic cell surface antigens. The results show (*a*) that lymphocytes from conventionally maintained rats are less reactive to human, hamster, guinea pig, and mouse cell surface antigens than to the major H alloantigens, and (*b*) that lymphocytes from germfree rats display no demonstrable reactivity to xenogeneic cells, but are quantitatively normal in their response to allogenic cells. The conclusion drawn from these observations is that the circulating lymphocyte pool of an individual consists of a greater proportion of cells reactive to H alloantigens of other members of the same species than to the xenogeneic cellular antigens of members of other species and that this large number of cells is not generated by a mechanism involving immunization to cross-reactive environmental antigens.

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