RESPONSIVENESS OF GERMFREE MICE IN MIXED LEUKOCYTE CULTURE

Reexamining the Nature of the Responding Unit*

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The major histocompatibility complex (MHC) in several species is highly polymorphic with several different alleles and many different antigens associated with each MHC. Studying the graft-*versus*-host (GVH) response in the chicken, in which lymphocytes respond to MHC differences, Simonsen noted that up to 3% of lymphocytes respond to a single difference in the MHC (1). A similar high frequency of responding units exists in the mixed leukocyte culture (MLC) test (2–4), a model of the recognition phase of the GVH reaction. In the MLC test, responding lymphocytes of one animal enlarge, exhibit enhanced incorporation of radioactive thymidine, and divide if they encounter MHC differences on allogeneic stimulating cells.

With the very great number of different alleles (and therefore antigens) associated with the MHC, possibly compounded by the existence of still different antigens to be recognized which are associated with xenogeneic MHC's, it has been suggested that the very high frequency of initially responding units in MLC is not consistent with clonal selection theory. Many different explanations have been offered to explain this apparent inconsistency. Most recently the suggestion has been made that the normal MLC response is directed only at allogeneic differences since xenogeneic responses in MLC were markedly lower than allogeneic ones using conventional (cv) animals (5) and further, germfree (gf) animals showed no xenogeneic response while responding normally in allogeneic mixtures (6). If the number of different allogeneic antigens which must be recognized is not too great, and only allogeneic antigens can cause MLC activation, these findings would help resolve the inconsistency. We have presented data which show that the xenogeneic and allogeneic responses in cv animals are in many cases comparable (7).

In the present paper we present data which show that a xenogeneic response can be obtained in gf animals. Our findings argue against the concept that the normal MLC response is aimed only at allogeneic MHC differences.

Mice used in this study were obtained from A. R. Schmidt, Madison, Wis. Two different strains of animals (C3H/He and HA/ICR) which were available as both gf and cv stocks were tested in each experiment. The gf status of mice was shown on the basis of both bacteriological

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studies and the absence of anti-viral antibodies in the serum. Control cv animals were matched with the gf animals for age and sex.

MLC tests were done using a micromethod recently described for human cultures (8, 9). Mouse responding cells were used at concentrations which give optimal response for allogeneic mixtures. All cultures were done in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with human plasma, penicillin, and streptomycin. Stimulating cells were treated with mitomycin C and tested at concentrations which gave maximal allogeneic stimulation or on preliminary testing good xenogeneic stimulation. Cultures were labeled with tritiated thymidine for 16 hr 3 days after the initiation of culture.

TABLE I

Mixed Leukocyte Cultures in Conventional and Germfree Mice

Responding cells	Stimulating cells					
	C3H/He	HA/ICR	Dog 1	Dog 2	Hu 1	Hu 2
A.						
C3H/He cv	(9874 ± 894)*	27,211 ± 317 $P < 0.010$‡	32,372 ± 10,479 $P < 0.010$	35,207 ± 4761 $P < 0.001$	26,162 ± 6360 $P < 0.005$	21,459 ± 499 $P < 0.001$
C3H/He gf	(7908 ± 2423)	28,555 ± 2776 $P < 0.005$	33,661 ± 2051 $P < 0.005$	45,439 ± 6759 $P < 0.001$	51,781 ± 4833 $P < 0.001$	15,832 ± 4884 $0.05 < P < 0.1$
HA/ICR cv	42,599 ± 5118 $P < 0.001$	(17,410 ± 669)	40,344 ± 4640 $P < 0.001$	34,176 ± 7620 $P < 0.010$	42.650 ± 9295 $P < 0.005$	35,477 ± 8709 $P < 0.010$
HA/ICR gf	65,640 ± 3001 $P < 0.001$	(15,441 ± 2256)	49,105 ± 4253 $P < 0.001$	36,319 ± 1789 $P < 0.001$	40,679 ± 9913 $P < 0.010$	35,653 ± 2723 $P < 0.001$
B.						
C3H/He cv	(9599 ± 1039)	25,993 ± 2087 $P < 0.001$	54,882 ± 2376 $P < 0.001$	33,074 ± 3940 $P < 0.001$	79,359 ± 8743 $P < 0.001$	39,566 ± 8873 $P < 0.001$
C3H/He gf	(6391 ± 2706)	21,327 ± 284 $P < 0.025$	61,264 ± 5188 $P < 0.005$	36,754 ± 1626 $P < 0.005$	68,736 ± 4715 $P < 0.005$	33,249 ± 9828 $P < 0.10$
HA/ICR cv	18,980 ± 1913 $P < 0.005$	(5654 ± 1299)	18,709 ± 2323 $P < 0.005$	12,877 ± 3124 $P < 0.025$	29,525 ± 6346 $P < 0.001$	13,463 ± 2516 $P < 0.010$
HA/ICR gf	25,422 ± 1671 $P < 0.001$	(3886 ± 1206)	72,841 ± 1970 $P < 0.001$	34,781 ± 5028 $P < 0.001$	74,848 ± 650 $P < 0.001$	35,623 ± 9330 $P < 0.001$

* Counts per minute ± standard deviation.

‡ P value for t test on log transformed data comparing each experimental mixture with the appropriate isogeneic control (given in parentheses).

We have done a total of four experiments in which both cv and gf mouse cells were tested for their response to allogeneic stimulating cells (either from cv or from gf animals) and xenogeneic stimulating cells. Xenogeneic cells from two humans and two dogs were included in each experiment. Results from two of the experiments are given in Table I. If we equate the counts per minute of radioactive thymidine incorporated into the allogeneic mixture to 100 and express all other stimulation values using that same responding cell as a percentage of 100, then in a summary of all four experiments the cv mouse response to human stimulating cells varied from 39.4 to 305.3% and the gf mouse response to human stimulating cells varied from 32.4 to 322.2%. The cv mouse response to dog stimulating cells varied from 26.1 to 211.1% and the gf mouse response to dog stimulating cells varied from 22.2 to 287.3%. These data demonstrate

that both cv and gf animals can respond to allogeneic and to xenogeneic cells, and that the degree of response of the cv and gf cells to a single allogeneic or xenogeneic cell varies somewhat but is of the same order of magnitude and certainly highly significant in most instances.

Whatever the basis of the allogeneic MLC response, we must conclude from these studies that such a response may also take place in xenogeneic combinations. These findings, with the more extensive xenogeneic tests done in cv animals (7), would not support the concept that cells responding in xenogeneic and allogeneic combinations represent different populations of cells (6).

There are several considerations which make the finding of the high frequency of the initially responding unit less dramatic than it may seem when compared directly with the antibody response to sheep red blood cells, for instance. First, we do not know how many different antigens must be recognized by the MLC responsive cell. It may be that there is extensive sharing of antigens, not only between different members of the same species but also between different species. Recent findings in mouse (10) and man (11–15) force a reevaluation of the basis of MLC stimulation. We have obtained evidence (10) that genetic differences of the MHC in the mouse, which by the usual methods of testing are serologically not detectable (and thus are at the very least difficult to determine serologically), and in some cases a locus (loci) of the MHC which is linked to, but genetically separable from the serologically defined loci, can result in MLC stimulation. These differences will be referred to as "antigens," even though no antibody has been obtained directed against them. It is possible that these lymphocyte-defined (LD) antigens are fixed in number, possibly even less than 100; and that the specificity of response to different allogeneic and xenogeneic stimulating cells is due to the relatively unique combination of a few of these LD antigens on each different population of stimulating cells.

Second, there is the possibility that one cell can respond to more than one antigen (pluripotency). It has been previously demonstrated that (at least at any one time) the responding cells in MLC are not totipotent. This evidence comes from studies in which the cells reactive to one allogeneic stimulating cell are eliminated from the culture with either 5-bromodeoxyuridine and light (16) or tritiated thymidine (17) and the remaining cells are shown to be no longer responsive to the same allogeneic cell stimulus but are responsive to other allogeneic cell stimuli. We do not know, however, whether the initially responding cells could have responded to other antigenic stimuli as well. These findings cannot, therefore, be used to argue that the responding cells are unipotent and thus leave open the possibility of pluripotency.

Pluripotency could be achieved in several ways. It may be that all the receptor sites of any one responding cell are molecularly identical. If the number of foreign antigens which must be recognized among all the allogeneic and xenogeneic stimulating cells is large (which we cannot be sure is true) the receptor molecule may be of relatively lesser specificity; several different antigens could

be recognized by this single receptor molecule. Alternatively it is possible that a given responding cell carries several different receptor molecules. The cell could either have the genetic information for all of these or the receptor molecules could be exchanged between responding cells so that some cells can respond via a receptor molecule which they themselves do not synthesize. It is also possible that each cell carries a totipotent battery of immune receptors. Phenotypic restriction of expression allowing for response specificity could be determined by a mechanism such as temporary geographic distribution of these receptors; that is, receptors might only be able to recognize an antigen and lead to an immune response if several receptors of identical specificity are grouped or "clustered" in particular regions on the cell surface.

The high frequency of initially responding units in the MLC has been dealt with above by considerations concerned either with the nature of the stimulating antigens or the responding cell potency. We have recently obtained data, referred to above in the discussion of the nature of the stimulating antigens, which may provide some unification for both these lines of explanation. Associated with the MHC are immune response (Ir) genes which control the level of immune response to a variety of antigens. It has been suggested that the products of these Ir genes may be the receptor molecules on thymus-derived (T) lymphocytes (18). The LD loci which are responsible for MLC map with and have not been genetically separated from the Ir loci. If the product of the Ir loci is the T cell receptor site, then it may be that the receptor molecule functions not only as the recognizing structure on the responding cell but also as the foreign antigen on the stimulating cell. However, not until we know how many Ir loci exist and have some measure of the degree of polymorphism at each, can we attempt any critical analysis of the possibility that the Ir loci actually provide the major genetic control for both response and stimulation.

SUMMARY

Conventional (cv) and germfree (gf) mice are able to give a good proliferative response to allogeneic cells in the mixed leukocyte culture (MLC) test, while the response to xenogeneic stimulating cells has been in question. Previous studies by others have suggested only a low MLC response in cv animals and none in gf ones. We have found that both cv and gf animals can give a good MLC response to xenogeneic as well as allogeneic cells. These findings are of importance for our understanding of both MLC stimulation and response.

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