QUANTITATIVE STUDIES ON THE MIXED LYMPHOCYTE INTERACTION IN RATS

II. Relationship of the Proliferative Response to the Immunologic Status of the Donors*

By DARCY B. WILSON,‡ Ph.D., WILLYS K. SILVERS,§ Ph.D., and PETER C. NOWELL,‖ M.D.

(From the Departments of Medical Genetics and Pathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104)

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On the basis of the results presented in the preceding communication (1), it was concluded that the DNA synthesis and proliferation, measured in terms of the incorporation of tritiated thymidine, which occurs when lymphocytes from two immunogenetically disparate rat donors are cultured together, represents an immunologic response at the cellular level. Positive culture responses did not occur unless the two cell donors differed at the important Ag-B histocompatibility locus (2). Indeed the occurrence of reactivity in mixed rat cell cultures from parental and *backcross* donors was dependent upon the existence of Ag-B antigenic differences between the donors—showing that the stimulatory agents of the proliferative response are histocompatibility antigens determined by this locus (1, 3).

The unlikely possibility was raised that the mitogenic factors of homologous cells are not Ag-B antigens, but rather are substances—not necessarily antigens—genetically determined by a locus closely linked to the Ag-B (1). The results of the present studies do not support such a possibility, but further suggest that the in vitro response has an immunological basis inasmuch as the presence or absence of proliferative responses—as well as their magnitudes—depend in great measure on the immunologic status of the cell donors.

Materials and Methods

The procedures and animal stocks involved in this study are fully described in the preceding paper (1).

Thymectomy: The thymuses were surgically extirpated from newborn (less than 24 hr after birth) BN rats using procedures described by Miller (4).

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‡ Fellow Helen Hay Whitney Foundation.

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Sensitization of some of the parental cell donors was accomplished by means of orthotopic skin homografts (5).

Tolerance was induced in neonatal animals by inoculation of homologous bone marrow suspensions intravenously (6). The recipients were test grafted with homologous skin approximately 6 wk later (5), and survival of the skin homografts with luxuriant hair growth for 50 days or longer was taken as a criterion of tolerance.

The conditions for culture were the same ones used in the previous studies (1). Blood lymphocytes were cultured in triplicate. Each culture contained a mixture of 1 million + 1 million lymphocytes from the two donors in 1 ml of culture medium. Control, unmixed cultures consisted of 2 million lymphocytes in the same volume.

Chromosome preparations were prepared according to the standard technique for leukocyte cultures described by Moorhead et al. (7), using either 0.75% sodium citrate or 0.075 M KCl as the hypotonic medium.

TABLE I

*Morphology of the Sex Chromosomes of Various Isogenic Strains of Rats**

Strain	♂	♀
BN‡	X_{ST}	$X_{ST}X_{ST}$
Lewis‡	$X_{ST}Y$	$X_{ST}X_{ST}$
Fisher	X_TY	X_TX_T
DA	X_TY	X_TX_T

* X_{ST}, X chromosome centromere subterminal; X_T, X chromosome centromere terminal; Y, morphologically identifiable Y chromosome (males of the BN strain have a Y chromosome, which is not distinguishable from the acrocentric autosomes).

‡ Hungerford and Nowell (8).

RESULTS

1. Further Studies on the Unidirectional Reactivity of Parental–F_1 Cultures.— Results of the preliminary chromosome studies presented in the preceding paper strongly indicate that the proliferative response which results when lymphocytes from parental DA and F_1 DA/BN rats are mixed and placed into culture primarily involves cells from the parental donor. To extend these observations, various parental–F_1 combinations were employed, selected on the basis that the sex chromosomes in mitotic figures of both donors, if present, are mutually distinguishable (8) (Table I). The results (Table II) show that (*a*) in cultures of cells from two unrelated F_1 donors (L/BN + DA/L), mitoses from *both* of the donors were present in approximately equal numbers; and (*b*) in mixed cultures from parental and F_1 animals, mitoses occur almost exclusively among cells of *parental* origin. A few (8/66) F_1 mitoses were seen in mixed cultures of L and DA/L cells, although the majority were of the parental type (58/66). In subsequent cell cultures with this parental–F_1 donor combination, DA/L F_1 cells comprised 5-28% of the mitotic figures on the 6th or 7th day of incubation. *Unmixed*, control cultures of DA/L cells also showed this activity regardless of

whether DA or BN serum was used as a medium supplement. Because of this background mitotic activity of F_1 cells in the control cultures, the F_1 mitoses in the mixed cultures did not seem to be related to any proliferative reactivity against parental cells. It is possible that these "nonspecific" late-appearing F_1 mitoses reflect a reactivity against the antibiotics or contaminating amounts of heparin (carried over from the wash fluid) present in the culture medium.

TABLE II

Identification of Proliferating Cells in Parental–F_1 Mixed Lymphocyte Cultures

Culture mixture and sex chromosome morphology		Distribution of mitotic figures and mitotic index				Totals
		Days				
		5	6	7	11	
L ♀ + DA/L* ♂	MI‡ (%)	<0.1	0.8	0.8	<0.1	
(X_{ST} $_{ST}$) + (X_TY)	$X_{ST}X_{ST}$	1	19	38	0	P 58
	X_TY	0	1	7	0	F_1 8
L/BN ♀ + L ♂	MI‡ (%)	1.0	2.8	2.6	<0.1	
($X_{ST}X_{ST}$) + ($X_{ST}Y$)	$X_{ST}X_{ST}$	0	0	0	0	F_1 0
	$X_{ST}Y$	1	9	49	2	P 61
F/BN ♀ + F ♂	MI‡ (%)	0.2	2.0	0.7	0.1	
(X_TX_{ST}) + (X_TY)	X_TX_{ST}	0	0	0	0	F_1 0
	X_TY	3	24	23	4	P 54
L/BN ♀ + DA/L ♂	MI‡ (%)	0.2	1.5	0.7	<0.1	
($X_{ST}X_{ST}$) + (X_TY)	$X_{ST}X_{ST}$	4	19	23	0	F_1(a) 46
	X_TY	8	15	14	0	F_1(b) 37

* F_1 parentage: ♀/♂

‡ Mitotic index: no. mitotic figures/100 nucleated cells after 4 hours colchicine, 1000 cells counted.

2. Reactivity of Cells from Immunologically Tolerant Donors.—To determine whether the immunologic status of the cell donors is reflected in the proliferative response of the mixed lymphocyte interaction, cells were obtained from parental donors made immunologially tolerant by inoculation at birth with homologous bone marrow cells.

Fig. 1 shows the results of culturing cells from Lewis donors tolerant of BN in the following combinations harvested at various times: (*a*) with BN tolerant of Lewis, (*b*) with L/BN, and (*c*) with L/DA. The results of this experiment are fully typical of many different combinations tested. Whereas the usual amounts of radioactive thymidine are incorporated into cultures of Lewis toler-

ant of BN cells mixed with cells from *indifferent* L/DA F_1 donors, little or no activity occurred in cultures of cells from mutually tolerant donors, or in cell cultures from Lewis tolerant of BN and L/BN F_1 animals.

3. Reactivity of Cells from Preimmunized Donors.—To determine what influence a preexistent state of transplantation immunity in the donor might have on the proliferative response of cells in the mixed lymphocyte interaction,

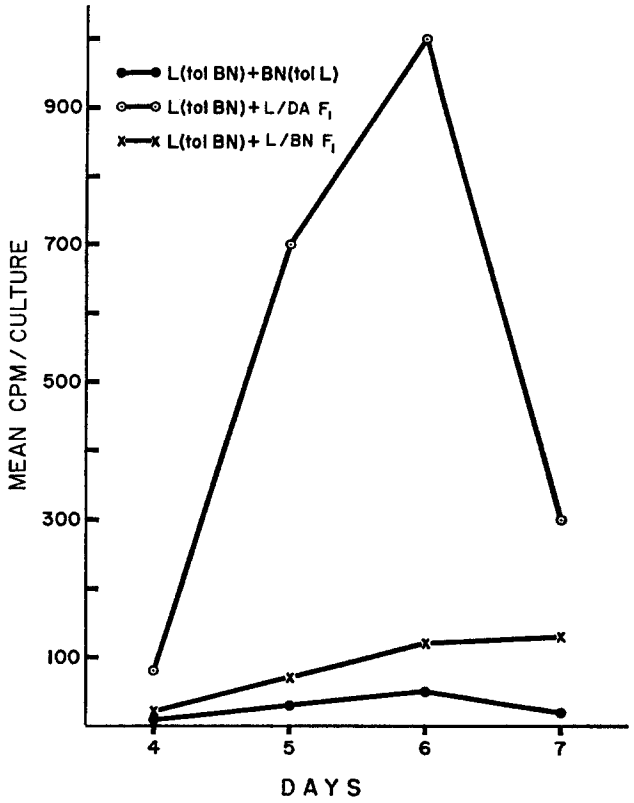

FIG. 1. Reactivity of cells from tolerant animals (see text).

peripheral blood lymphocytes from L/BN animals were cultured with lymphocytes from *normal* or preimmunized Lewis strain donors. The immunized animals had been grafted bilaterally 10 days earlier with skin from rats of the BN strain.

The results of this experiment (Fig. 2) were somewhat surprising, but clear-cut, and the same pattern of response was revealed with repeated experiments. During the 2nd and 3rd days after initiating the cultures, the uptake of thymidine was slightly greater in cultures of presensitized Lewis + L/BN cells. The

response in these cultures for the next few days was similar to that obtained with normal cells, but then it declined rather abruptly while the normal cells continued to proliferate. Culture inocula consisting of lymphocytes obtained from the lymph nodes of normal or sensitized Lewis donors with L/BN lymph node cells gave similar results.

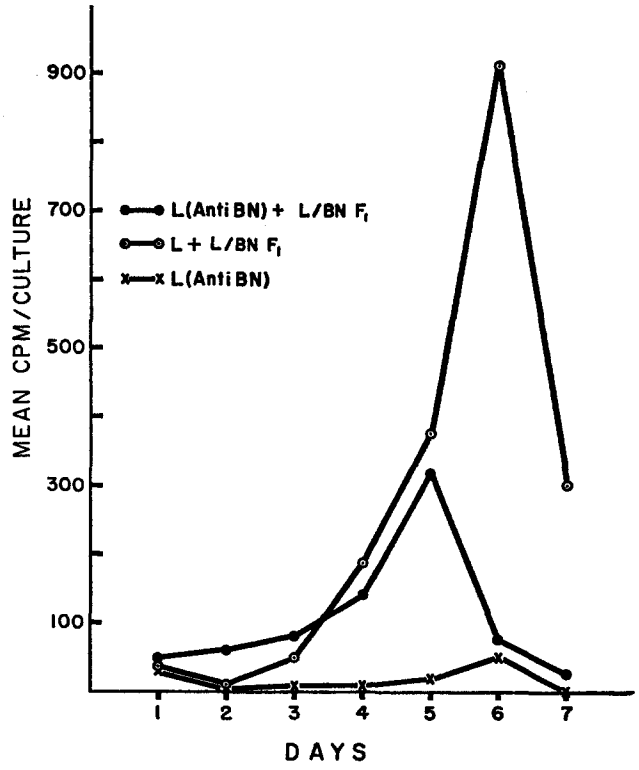

FIG. 2. Comparison of reactivity of Lewis cells from normal or presensitized donors against L/BN hybrid cells.

Experiments were conducted with peripheral blood and lymph node lymphocytes from animals of the Fischer strain that had been grafted with Lewis skin. Similar to the results of cultures of normal cells from this strain combination, mixtures of cells from preimmunized Fischer and normal Lewis donors did not show any incorporation of radioactive thymidine. Since animals of both these strains bear the Ag-B^1 histocompatibility antigen, this finding clearly indicates that even with the use of culture inocula from preimmunized donors proliferative responses in the mixed lymphocyte interaction do not occur unless the codonors are of a different Ag-B genotype.

4. Response of Cells from Thymectomized Animals.—Extensive immunologic defects are known to develop in rats thymectomized at or shortly after birth. Results of numerous studies have suggested that such basic cellular processes as lymphoid proliferation and maturation may be dependent upon an intact thymic function in vivo (9). To determine whether the thymus has any influence on the capacity of cells to proliferate in the mixed lymphocyte interac-

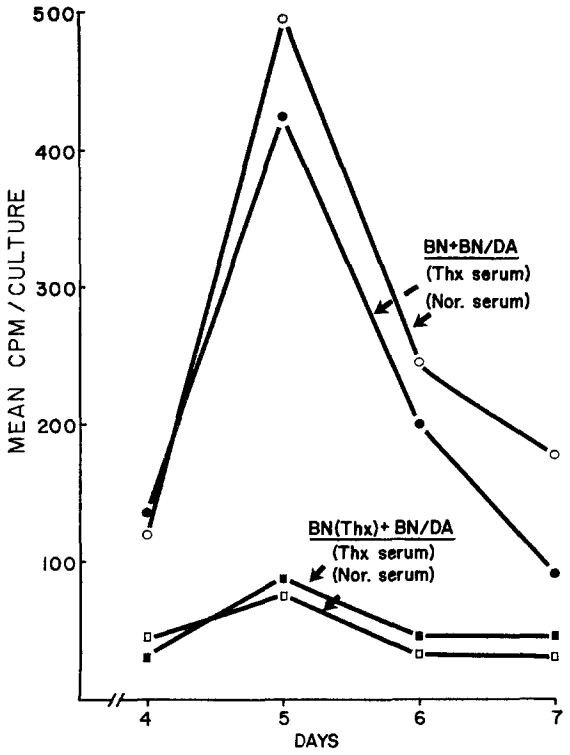

FIG. 3. Comparative reactivity of cells from normal or neonatally thymectomized BN donors against BN/DA cells. Whether or not sera from thymectomized donors is used to supplement the culture media has no effect on the response of normal BN cells.

tion, peripheral blood lymphocytes were obtained from thymectomized parental strain donors and committed to culture with cells from hybrid animals.

The results of a typical experiment are shown in Fig. 3. Responses in cultures consisting of lymphoid cells from thymectomized BN + normal BN/DA animals were strikingly less than cultures containing cells from unoperated BN and F_1 rats. It should be emphasized that each of these cultures contained a similar number of parental strain lymphocytes, whether from normal or thymectomized animals, and therefore the nonresponsiveness of cells from thymectomized

donors cannot be attributed to differences in numbers of circulating lymphocytes as a result of thymectomy. Thus the data strongly suggest that on a cell-for-cell basis, lymphocytes from thymectomized donors are markedly debilitated in their response to homologous transplantation antigens in the mixed lymphocyte interaction.

It was also of interest to determine whether BN serum obtained from thymectomized animals was any less effective in supporting the proliferation of parental strain BN cells to BN/DA lymphocytes. This was achieved by comparing the response in mixed cultures of normal BN + BN/DA lymphocytes in medium supplemented (15%) with serum from normal or thymectomized animals; no differences were observed.

DISCUSSION

It is well known that the degree of immunologic reactivity in the intact animal is greatly dependent upon its immunologic status. Thus when confronted with a foreign antigen, neonatally *thymectomized* animals show a markedly decreased response (10), immunologically *tolerant* hosts exhibit a nonresponsiveness which is specific for the antigen(s) to which they have been made tolerant (11), F_1 *hybrid* hosts do not react immunologically to grafted tissues of parental origin (12), and *presensitized* animals respond sooner and with more vigor upon rechallenge (13).

As shown in these experiments, the proliferative response of cells in the mixed lymphocyte interaction is also greatly influenced by the immunologic status of the cell donors and this provides further evidence that the reaction has an immunologic basis. Thus (*a*) lymphocytes from *thymectomized* donors display a diminished proliferative reactivity when cultured with homologous cells; (*b*) lymphocytes from immunologically *tolerant* donors exhibit a nonreactivity which is specific only to cells bearing the homologous antigens of the tolerance-inducing strain; (*c*) cells from F_1 *hybrid* animals do not react to parental strain antigens; and (*d*) lymphocytes from presensitized animals exhibit a curtailed proliferative activity in mixed cultures with cells from donor strain animals to which they have been immunized. Indeed, except for the last finding, these observations are consistent with the criteria by which certain adaptive responses in vivo are judged to have an immunologic basis.

A possible explanation for the one exception mentioned is that the curtailed proliferative reactivity in cultures of cells from preimmunized animals may reflect an immunospecific destruction of the stimulating cells by the sensitized lymphocytes. Some support for this premise is provided by the fact that lymphocytes from sensitized animals are fully capable of destroying and arresting the growth of monolayer cultures of homologous "target" fibroblasts (14, 15). Also, the finding that proliferation in mixed cultures of cells from certain parental-F_1 donor combinations is *greater* than with the parental-parental

mixtures suggests that mutual stimulation may be accompanied by the appearance of immunologically destructive capacities by the co-cultured cells. If such a destructive effect does occur, it could interfere with DNA synthesis by reducing the number of stimulatory target cells as well as by curtailing metabolic activities of reactive cells.

Rieke recently suggested that the immunologic deficiency of cells from thymectomized animals, e.g., their failure to produce runt disease when inoculated into newborn homologous hosts, might reflect an inability on their part to undergo a proliferative response. However, he was able to show that at least some cells from thymectomized rat donors underwent blastogenic transformation and mitosis when exposed to phytohemagglutinin, or lymphoid cells of heterologous (murine) or homologous origin. These findings are not necessarily inconsistent with the data presented herein on the behavior of cells from thymectomized animals. Rieke's findings suggested that there may be a quantitative difference in the proliferative behavior of cells from thymectomized donors, however, they were not conclusive (16).

The behavior of cells in the mixed lymphocyte interaction would be difficult to explain on any basis *other* than an immunologic one. That some nonimmunologic process based on mutual contact by cells with structurally different surface patterns (17, 18) is not involved is clearly shown by the selective nonresponsiveness of cells from tolerant donors against homologous cells bearing specific transplantation antigens and the generalized nonreactivity of cells from thymectomized donors. Rather, these findings clearly indicate that some kind of immunologically specific recognition process is involved in initiating the proliferative phase of the mixed lymphocyte interaction. That the proliferative phase itself, is immunologically specific is also shown by the findings that even in an environment of proliferating parental cells, F_1 cells themselves are not induced to respond. This rules out the possibility that a specific immunologic reaction releases into the medium some product which nonspecifically initiates a proliferative response.

The cell type which responds in the mixed lymphocyte interaction is apparently the small lymphocyte. The immunologic competence of this cell in situations of graft-versus-host reactivity is now well established (see reviews, references 19, 20). It is known that inocula consisting almost entirely of small lymphocytes, derived from parental strain donors, can induce a fatal wasting disease in hybrid hosts. One of the earliest events that has been described is a transformation of donor small lymphocytes into large pyroninophilic cells in the splenic tissue of the host animals. Since lymphocytes from tolerant donors neither transform into pyroninophilic cells nor induce the slightest symptoms of runt disease, it is believed that cell transformation by small lymphocytes represents an essential part of the initial phases of the primary immunologic response to transplantation antigens (19). Consistent with this premise are the present findings that lymphocytes from tolerant animals are also incapable of

responding to specific transplantation isoantigens in vitro, even though they are fully responsive to the presence of third party homologous lymphocytes.

From their studies demonstrating the inability of pure suspensions of lymphocytes from tolerant donors to produce runt disease in F_1 hosts, Gowans and his co-workers further suggested that the immunologic deficiency of a tolerant animal resides with the lymphocyte population and not with macrophages or other cell types (19). While the results of the present experiments with cells from tolerant animals are in accord with this conclusion, they do not answer the further question as to whether an animal's state of tolerance depends on the *presence* of tolerant, nonreactive lymphocytes or on the *absence* of all potentially reactive cells.

Finally, the results of these experiments add additional evidence to the premise that lymphocytes may not require a period of residence within the architecture of a lymph node or the presence of other cell types in that tissue to initiate an immune reaction. In a recent, carefully documented study, Elkins and Palm (2) concluded that of the many histocompatibility loci present in rats, only one—the Ag-B—is involved in the elicitation of localized renal graft-versus-host reactions. When immunologically competent lymphocytes from parental strain donors were inoculated beneath the renal capsules of backcross recipients, renal lesions developed only in animals that could be shown by serologic procedures to possess an Ag-B antigen not present in the inoculated cells. These findings, together with the evidence presented here on the mixed lymphocyte interaction, suggests that in certain situations such as in culture, or in local cellular deposits, where the full complement of cells involved in immunologic response mechanisms required for antigen processing—macrophages, reticuloendothelial cells, lymphocytes, etc.—are not present, only the "strong" histocompatibility antigens can be detected. It is possible that immune responses to these antigens depend on their intrinsic immunogenicity, or that they have the unique property to stimulate lymphocytes directly without the involvement of other cell types.

SUMMARY

The influence of the immunologic status of the cell donors on the proliferative behavior of rat lymphocytes in the mixed lymphocyte interaction has been studied.

Mixed cultures of cells from various parental and F_1 combinations having morphologically distinguishable sex chromosomes exhibited unidirectional proliferative reactivity. The mitotic figures were predominately of parental origin.

Lymphocytes from donors made tolerant at birth to homologous transplantation isoantigens were specifically unreactive against cells bearing antigens of the tolerance inducing strain, but not to indifferent third party homologous lymphocytes.

Cells from animals that had been surgically thymectomized at birth exhibited

a markedly and sometimes totally diminished reactivity against homologous lymphocytes.

Presensitization of the cell donors resulted in a curtailment of proliferative reactivity in cultures with cells bearing the immunizing antigens. This may reflect the destructive properties that lymphocytes from sensitized animals are known to possess.

The results of these experiments show that the proliferative activity of lymphocytes in the mixed lymphocyte interaction accurately reflects the immunologic status of the cell donors, and these findings provide further support for the premise that the mixed lymphocyte interaction represents a primary immunologic response by cells in culture against homologous cells bearing histocompatibility antigens.

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