QUANTITATIVE STUDIES ON THE MIXED LYMPHOCYTE INTERACTION IN RATS

V. Tempo and Specificity of the Proliferative Response and the Number of Reactive Cells from Immunized Donors*

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Extensive studies of the mixed lymphocyte interaction (MLI)[1] have shown it to be an immunologically specific proliferative reaction in which a large proportion of immunologically competent, predominately thymus-derived, circulating lymphocytes from nonimmunized donors recognize and respond to the presence of major histocompatibility (H) isoantigens in culture (1–7). Prior immunization of the cell donors is known to alter the time course of the subsequent proliferative response in the MLI (2, 3, 8) but little is known of (*a*) the specificity and (*b*) the duration of the altered proliferative capacity, (*c*) the influence of different routes of antigen administration, and (*d*) whether the proportion of potentially responsive lymphocytes is also changed.

In the present study, we undertook an examination of these various parameters with the following results: (*a*) Subcutaneous immunization accelerates the tempo of the proliferative response, whereas antigen given systemically decreases it. (*b*) The altered proliferative behavior is specific for the immunizing antigens. (*c*) The period after immunization during which the MLI displays an altered tempo is comparatively short-lived, lasting not longer than 2–3 wk. (*d*) The same proportion of lymphocytes are stimulated to respond in the MLI whether they are derived from immunized donors or from normal donors, even though the response profile is different.

Materials and Methods

Animals.—The animals used in this study were rats of the highly inbred Lewis (L), Brown Norway (BN), DA, and Black Hooded (BH) strains and their F_1 hybrids. Animals of the L

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[1] *Abbreviations used in this paper:* GVH, graft-*versus*-host; H, histocompatibility; HBSS, Hanks' balanced salt solution; ^{3}H-TdR, tritiated thymidine; MLI, mixed lymphocyte interaction.

and BH strains share the same major H isoantigen system (AgB-1); the other strains differ at this locus (9, 10, and footnote 2).

Immunizations.—Accomplished by injection of 30–40 million splenic cells in 1 ml of Hanks' balanced salt solution (HBSS) intravenously, intraperitoneally, or subcutaneously (bilaterally) in the axillary regions. The splenic cell suspensions were prepared by mincing the tissue and passing it through a 50 gauge mesh screen (11).

Cultures.—Conducted with cells from parental strain and F_1 donors by procedures fully described elsewhere (1, 4). Blood was collected by cardiac puncture at various times after immunization, leaving the donors alive. Leukocyte suspensions, consisting mostly of lymphocytes, were prepared by dextran sedimentation; the cells were washed, suspended in medium, and cultured at a concentration of 2 million per ml, 1 million from each of the co-donors.

Proliferation was assessed by the incorporation of tritiated thymidine (^{3}H-TdR) over a 16 hr period. The cultures were terminated at various times and the activity measured by liquid scintillation spectrometry (4).

Enumeration of the proportion of peripheral blood lymphocytes capable of being stimulated by a given H isoantigen system in the MLI was accomplished by a modification of methods described previously (4). The rationale of these procedures stems from the finding that not all the cells destined to respond in the MLI do so at the same time; rather, a given potentially responsive, but hitherto mitotically inactive, cell may enter into the DNA synthetic phase (S) for the first time at any time between 40 and 140 hr of culture (4). These newly dividing cells, entering the S phase for the first time, can be detected by pulse labeling with ^{3}H-TdR and radioautography. Cell division and the production of daughter cell progeny, which are also reactive in the MLI, can be prevented without inhibiting DNA synthesis of the initially reacting cells by including colchicine in the culture medium (final concentration, 5×10^{-6} M) from the outset. Thus only first division cells are detected.

At various times after initiating the cultures, duplicate tubes of each culture combination were exposed to ^{3}H-TdR for a period of 4 hr. The cultures were then terminated and air-dried slides of the cells prepared according to standard techniques for chromosome preparations using 0.75% sodium citrate as the hypotonic medium (12). Radioautographs were made by the dipping method with Kodak NTB-2 emulsion. The slides were exposed for a period of 10 days in light-tight boxes at 4°C after which they were processed in Kodak D-19 Developer Rapid Fixer, and water rinses. The cells were stained through the emulsion with Giemsa.

The number of reactive cells which entered the proliferative cycle on an hourly basis at, a given time in the culture period was determined from the following formula:

$$\begin{matrix}\text{No. reactive} \\ \text{cells per hour}\end{matrix} = \begin{matrix}\text{No. labeled cells} \\ \text{per 1000 cells}\end{matrix} \times \begin{matrix}\text{No. surviving} \\ \text{cells per culture}\end{matrix} \times \frac{1}{S + p}$$

where S = the length of the DNA synthetic S period in hours, and p = the number of hours the cultures were exposed to ^{3}H-TdR (4).

Duplicate radioautographs were prepared from each culture providing four slides for analysis of any one point in time. The proportion of labeled nuclei, including labeled mitotic figures, per 2000 cells was determined for each of the four slides. The number of surviving cells per culture was determined on parallel cultures with a Coulter particle counter (Coulter Electronics, Hialeah, Fla.) at the same time periods that the labeled cultures were terminated. Nuclear isolates were prepared by pronase digestion and exposure to cetrimide, as described by Stewart and Ingram (13).

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[2] Wilson, D. B., and J. Palm. Manuscript in preparation.

Cells exposed to ^{3}H-TdR become labeled at any point in the duration of their S phase, and according to these procedures, they will be scored as labeled cells while they are in the S phase and also as they pass through G_2 and accumulate as labeled mitoses. Consequently, the number of labeled nuclei per culture is divided by the sum of the S phase (6 hr, see reference 4) and the pulse period (p = 4 hr) to provide an estimate of the number of responsive cells per culture per hour.

This procedure is repeated at various times during the culture period, and the data plotted as "newly dividing" cells per hour *versus* hours of culture. The area under the curve provides an estimate of the number of peripheral blood lymphocytes in the original culture inoculum which are stimulated to proliferate in the MLI (4).

RESULTS

Influence of Prior Immunization Against Strong or Weak H Differences on the Proliferative Behavior of Lymphocytes in the MLI.—Numerous studies have recorded the time course of the proliferative interaction in the MLI, measured in terms of the incorporation of ^{3}H-TdR into newly synthesized DNA using lymphocytes from normal, presumably nonimmunized donors (4). In cultures consisting of parental strain rat peripheral blood lymphocytes stimulated with F_1 cells, the earliest indication of DNA synthesis is at 2 days; it increases during the 3rd and 4th days, and reaches peak values during the 5th or 6th days of culture.

To determine what influence immunization of the parental cell donors might have on the tempo of this response, groups consisting of three BH rats each were injected subcutaneously with 30 million splenic cells of BN (AgB incompatible), L (AgB compatible), or as normal controls of BH origin. The inoculated animals were bled 7 days later, and the following culture combinations initiated: (*a*) BH (sens BN) + BH/BN, (*b*) BH (sens L) + BH/L, and (*c*) BH (control) + BH/BN, and (*d*) BH (control) + BH/L. Cultures were exposed to ^{3}H-TdR and terminated on days 1–5 of culture.

The results of one experiment are presented in Fig. 1 as cpm/culture for each culture combination. For comparative purposes, an index of immunization is also plotted; this consists of the ratio of ^{3}H-TdR incorporation values in mixed cultures of cells from sensitized donors (sensitized MLI) to the values obtained with comparable mixed cultures of cells from nonimmunized donors (normal MLI), i.e., group (*a*)/group (*c*) and group (*b*)/group (*d*). An index of 1.0 indicates that lymphocytes from sensitized donors are neither more nor less responsive to specific F_1 cells than lymphocytes from control animals inoculated with isologous splenic cells. Each point in the figure represents the mean value of triplicate cultures from each of three animals (nine cultures). This kind of experiment was performed several times with different strain combinations and at various times after immunization.

The data presented in Fig. 1 are typical of results obtained with cells in mixed cultures initiated 7–10 days after immunization. The findings suggest the following conclusions: (*a*) Donor combinations incompatible with respect to the major antigens of the AgB locus are always responsive, and prior subcutaneous

immunization markedly accelerates the tempo of the proliferative response. During the 1st and 2nd day of culture, there is a 1.5–5-fold increase in ^{3}H-TdR incorporation over control values for cultures from nonimmunized donors. This is followed by a decrease in responsiveness to the point where cells from im-

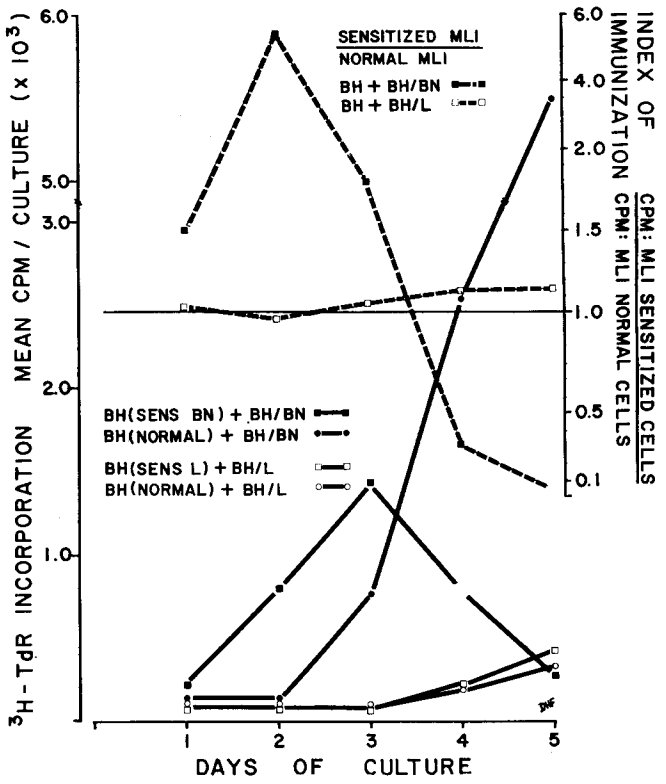

FIG. 1. Proliferative response of lymphocytes from normal BH strain rats or BH rats previously immunized with AgB-incompatible BN, or AgB-compatible L strain splenic cells in the MLI (lower curves). The upper curves represent the ratio of ^{3}H-TdR incorporation, cpm in sensitized MLI to cpm in normal MLI. Increased levels of proliferation occur in the MLI as the consequence of subcutaneous immunization with homologous cells differing with respect to major, AgB, histocompatibility antigens.

munized donors in 5-day cultures may display only 5% of the reactivity of comparable cultures of cells from normal donors. (*b*) Culture combinations in which the donors are compatible at the AgB histocompatibility locus, even when they differ by multiple weak H isoantigens, display no proliferative reactivity above values for nonstimulated cultures (not shown in Fig. 1) whether the cells were derived from normal or previously immunized animals.

Specificity of the Accelerated Proliferative Response in the MLI with Cells from

Immunized Donors and Influence of the Route of Antigen Administration.—The following experiment was designed to provide information on the influence of different routes of antigen administration and on the specificity of the accelerated proliferative response in the MLI with cells from immunized animals.

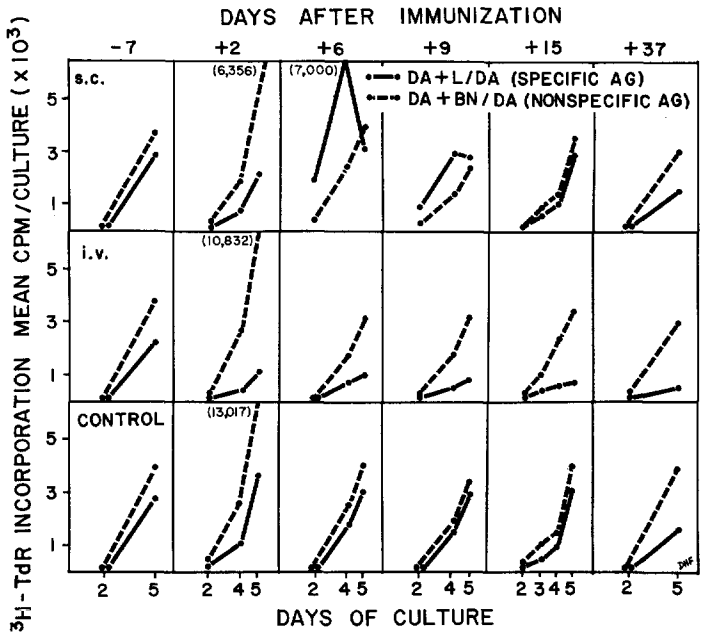

FIG. 2. Specificity of the altered tempo of the proliferative response in the sensitized MLI (cpm *versus* days of culture). Groups of DA strain rats were immunized subcutaneously or intraveneously with 40 million L strain splenic cells. An additional control group was inoculated with DA splenic cells. Cultures were initiated at various times, before and after immunization, with F_1 leukocytes from L/DA donors (bearing the specific, immunizing H isoantigens) or from BN/DA donors (nonspecific antigens). Subcutaneous immunization resulted in an accelerated proliferative response in the MLI specific for the immunizing antigens, whereas intravenous immunization specifically decreased the proliferative response.

Groups each consisting of three DA rats were inoculated with 40 million Lewis splenic cells subcutaneously or intravenously. For comparative purposes, a control group of DA rats was inoculated subcutaneously with DA splenic cells. All animals were bled 7 days before immunization and 2, 6, 9, 15, and 37 days after immunization. The cells were cultured with leukocytes from L/DA (specific antigen) and from BN/DA donors (nonspecific antigen). Cultures were exposed to ^{3}H-TdR and terminated 2–5 days after they were initiated. This experiment was conducted one other time with the same strain combination and two other times with a different strain combination with the same results. In addition the effect of prior intraperitoneal immunization was investigated and found to be the same as with intravenous immunization.

As before, the results are presented in terms of ^{3}H-TdR incorporation in mean cpm/culture

(triplicate cultures per animal, three animals per group; hence each point represents nine cultures) in Fig. 2, and as an index of immunization, the ratio in cpm of sensitized MLI to normal MLI, in Fig. 3.

Figs. 2 and 3 show that lymphocytes obtained from the various groups of animals before their immunization were mutually consistent in their proliferative

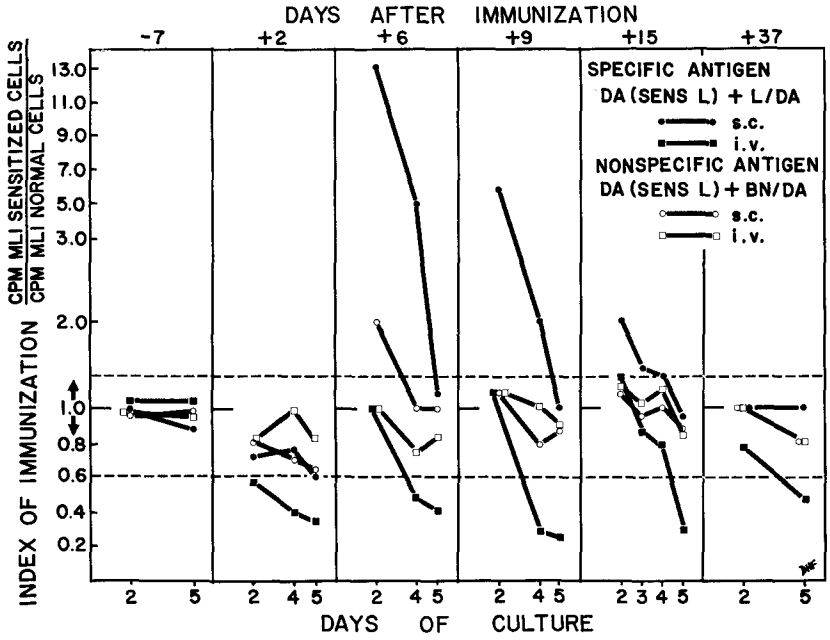

FIG. 3. Index of immunization. The data plotted in Fig. 2 are replotted here as the ratio in cpm of sensitized MLI to normal MLI. Closed circles or squares (●, ■) represent cultures stimulated with specific L/DA F_1 cells bearing immunizing H isoantigens; open circles or squares (○, □) represent cultures from the same normal or presensitized DA donors stimulated with indifferent BN/DA F_1 cells.

response to homologous H isoantigens; 2-day cultures displayed insignificant levels of activity whereas 5-day cultures of DA lymphocytes stimulated with L/DA and with DA/BN leukocytes were reactive to the extent of approximately 2600 and 3800 cpm, respectively.

2 days after immunization, cells from both intravenously and subcutaneously immunized animals were markedly less reactive to the specific antigen-bearing (L/DA) cells than the control inoculated group. This was especially pronounced with intravenously immunized donors, and it was specific for the immunizing antigen. The same cells cultured with DA/BN cells, bearing nonspecific antigens, were more nearly normal in their response, although there was some reduction in reactivity.

Cultures initiated 6 days after immunization displayed a different response profile. Lymphocytes from subcutaneously immunized animals were highly reactive (13- and 5-fold increase in 2- and 4-day cultures) and lymphocytes from intravenously immunized animals were much less reactive (more than 50% decrease) than cells from control inoculated animals when stimulated with the specific antigens. When these same cells were stimulated with nonspecific antigens, they displayed a more normal pattern of response, although there was a moderate increase (2-fold) in activity of subcutaneously immunized lymphocytes against the nonspecific antigen-bearing lymphocytes.

Cultures established 9 and 15 days postimmunization continued to display an altered tempo of reactivity; subcutaneously immunized lymphocytes were reactive much earlier than nonimmunized cells, and intravenously immunized lymphocytes were markedly less reactive. This response profile was specific for the immunizing antigens; cultures stimulated with cells bearing the indifferent isoantigen did not differ from normal.

The accelerated proliferative response of lymphocytes from subcutaneously immunized animals did not prove to be a long-lasting phenomenon; at 37 days postimmunization the response of subcutaneously immunized cells was quite normal. On the other hand, lymphocytes of intravenously immunized animals were still markedly and specifically less reactive.

Comparison of the Proportion of Reactive Lymphocytes in the MLI with Cells from Normal and Previously Immunized Donors.—The accelerated proliferative behavior of lymphocytes from subcutaneously immunized animals might reflect an underlying increase in the proportion of specifically reactive cells in the circulating lymphocyte pool. This possibility was tested in the following experiment.

A group of DA rats was immunized subcutaneously with 40 million BN splenic cells. 7 days after injection peripheral blood leukocyte suspensions were prepared from each of these animals and from an additional panel of three normal DA rats. Mixed cultures from each of these suspensions were established against DA/BN cells. The proportion of reactive lymphocytes from each of the donors was determined according to the procedures outlined above (see Materials and Methods).

The data obtained from each rat within the two panels were pooled and the mean number of reactive cells per hour at a given hour of culture is plotted in Fig. 4. The vertical bars represent the standard error of the mean. Two slides were prepared from each culture, the cultures conducted in duplicate, each panel consisting of 3 animals; hence each point on the curve represents the mean of 12 determinations.

The results show that the accelerated proliferative response of cells from immunized animals as measured in terms of ^{3}H-TdR incorporation was accompanied by a shift in the time when most new cells enter the proliferative cycle for the first division. Whereas with the lymphocytes from normal animals the

greatest number of newly dividing cells on an hourly basis occurred at approximately 80 hr of culture, with lymphocytes from previously immunized donors, the maximum numbers entered the proliferative cycle at approximately 45 hr of culture. However, the total number of responsive cells from normal or from immunized animals was approximately the same, 3.1% and 2.8% respectively

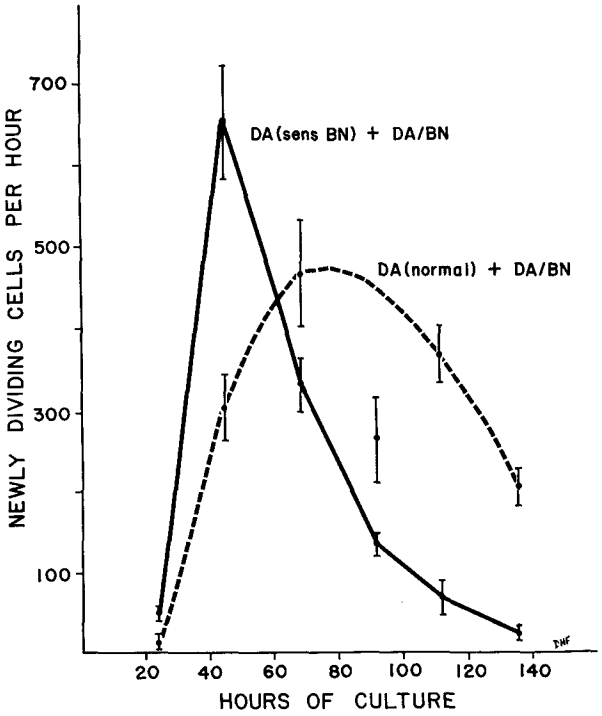

FIG. 4. Number of newly dividing parental strain DA lymphocytes per hour from normal or previously immunized donors in the normal or sensitized MLI. The area under each of the curves provides an estimate of the total number of responsive lymphocytes among the 1 million parental strain lymphocytes present when cultures were initiated. A similar proportion (approximately 3%) are responsive whether or not the donors were previously immunized.

of the initial number committed to culture, as determined by a comparison of the areas under the curves in Fig. 4.

DISCUSSION

These experiments demonstrate that with rat strain combinations incompatible at the major AgB histocompatibility locus, prior immunization markedly influences the behavior of lymphocytes exposed to the immunizing antigens in the mixed lymphocyte interaction. Immunization via the subcutaneous route

accelerates the proliferative response, whereas antigen administered systemically (intravenously or intraperitoneally) decreases it. This altered response profile is specific for the immunizing antigen(s), is comparatively short-lived, and is not accompanied by an increase in the proportion of reactive cells over that already present in the circulating lymphocyte population of normal animals.

The finding that prior immunization does not increase the proportion of reactive lymphocytes in the MLI provides a direct confirmation of conclusions reached by Simonsen and his colleagues in their studies with a graft-*versus*-host (GVH) system of the "factor of immunization" (14, 15). They demonstrated that immunization of lymphocyte donors with respect to a major H isoantigen system did not markedly alter the quantitative capacity of these cells to produce a GVH reaction. From the experiments in vivo with a GVH system (14, 15), and from our own studies in vitro with the MLI (4), it would appear that the number of lymphocytes reactive to a given H isoantigen system is already a substantially large one and cannot be increased significantly by immunization.

This conclusion prompts the suggestion that the cellular basis of enhanced immunological reactivity (memory) to strong H isoantigens is operationally different from that for both humoral and cell-mediated immunities to environmental antigens. In these immunities a significant proliferative amplification of clones of antigen-reactive cells is induced by exposure to antigen. In contrast, immunization to the strong H isoantigens does not increase the number of reactive cells but does, temporarily at least, significantly reduce the latent period for the cells to be stimulated by antigen. This suggests that memory for immune reactivity to strong H isoantigens may involve a temporary period when antigen-reactive cells are primed to respond to the specific antigen (16).

One argument which has been invoked as an explanation of the surprisingly large number of reactive cells in the MLI and the GVH reaction is that they may be multipotential and possess the capacity to react to more than one H isoantigen system (14, 17). There are several findings which argue strongly against the concept of multipotentiality: (*a*) the present studies that cells from rats immunized subcutaneously display an accelerated tempo of proliferation, but only when cultured with F_1 leukocytes bearing the specific immunizing antigens, and display normal reactivity against F_1 cells with a different set of H isoantigens; (*b*) The well established cytodestructive effects of lymphocytes from immunized donors to target fibroblast monolayers require that the fibroblasts possess the immunizing antigens and not a different set (18); (*c*) Previous studies with the MLI, that the magnitude of the proliferative response is increased additively when the cells are exposed to two different antigen systems simultaneously (5); and (*d*) A state of immunological tolerance induced to one antigen system does not influence the response to a different antigen in the MLI (5). These observations provide strong, although certainly not conclusive, evi-

dence for a model in which separate subpopulations of lymphocytes are responsive to different H isoantigens.

With respect to the lack of response in the MLI to weak antigen systems, a tentative explanation has been offered previously. Weak H isoantigens may constitute a negligible proliferative stimulus simply because the frequency of potentially reactive lymphocytes for these antigens is much smaller than the frequency of lymphocytes responsive to the major H isoantigens (5). Along these lines, it has been suggested that this difference in frequency of reactive cells may constitute the biological basis for the distinction between strong and weak transplantation antigens in vivo (5). From this reasoning, it would be expected that donors previously immunized to a weak antigen system possess an expanded clone of reactive lymphocytes and would therefore display a significant proliferative response when their cells were subsequently stimulated with these antigens in culture. Apparently this is the case with respect to the tuberculin antigens; lymphocytes from nonimmunized donors do not react in culture with PPD, but do react if the donors are previously immunized. Furthermore, rat ymphocytes are far more reactive to heterologous leukocytes if they have been obtained from previously immunized donors (5).

These expectations, however, were not fulfilled with immunization to the weak H isoantigens in our present experiments, and no adequate explanation is obvious. It may be possible that an expanded population of lymphocytes does accompany immunization with these antigens, but that it is not to be found among the circulating lymphocyte pool in the peripheral blood. Another possibility might be that immunization with weak H isoantigens provokes the development of a humoral immunity which in turn inhibits the production of significant numbers of circulating thymus-derived lymphocytes reactive to these antigens. Similar explanations might be offered for the reduced MLI reactivity of lymphocytes from animals immunized via the intravenous route.

SUMMARY

These studies were designed to determine what influence prior immunization with homologous H isoantigens might have on the subsequent proliferative activity of lymphocytes from these animals in the mixed lymphocyte interaction. The results demonstrate the following: (*a*) Subcutaneous immunization with splenic cells from donors differing at the major H locus accelerates the tempo of the proliferative response to F_1 cells bearing these same antigens in the MLI, whereas antigen given systemically reduces the proliferative response. (*b*) The altered proliferative behavior is specific for the immunizing antigens. (*c*) The period after immunization during which the MLI displays an altered tempo is a short one, lasting not longer than 3 wk. (*d*) Whether they are derived from previously immunized or from normal donors, the proportion of lymphocytes responsive in the MLI is the same, even though the response profiles are different.

These results suggest that in comparison to immune responses to other types of antigens, immunologic reactivity to the major H isoantigens already involves a large number of antigen-reactive cells in normal animals and that the proportion of these cells is not increased as a result of immunization. Rather, lymphocytes from immunized animals respond more rapidly to the presence of these antigens.

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