CELLULAR BASIS OF THE GENETIC CONTROL OF IMMUNE RESPONSES TO SYNTHETIC POLYPEPTIDES

I. Differences in Frequency of Splenic Precursor Cells Specific for a Synthetic Polypeptide derived from Multichain Polyproline ([T, G]-Pro--L) in High and Low Responder Inbred Mouse Strains*

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Genetic controls of immune responses to natural and synthetic antigens are expressed by quantitative, autosomal traits (1). A number of separate dominant genes have been identified which regulate the ability of inbred strains of mice and guinea pigs to elicit specific immune responses to these immunogens (2–13). Although two genetic loci have been shown to be linked to histocompatibility regions of the mouse genome (12, 14, 15), little is known concerning the genetic mechanisms responsible for the control of immunity. In view of the number of complex cellular events required for immune processes, it is likely that at least certain genetic controls will be demonstrable at the cellular level. In fact, responses of mice to synthetic polypeptides based on multichain polyalanine and responses of guinea pigs to poly-L-lysine have been achieved by the injection of responder lymphoid cells into irradiated nonresponder animals (1, 14). Such transfer experiments suggest that these genetic defects reside in potentially immunocompetent cells, although the nature of the cellular deficiency is not fully understood. Previous results indicate that the so-called "nonresponder" mice do produce small amounts of antibody to poly-L-(Tyr, Glu)-poly-L-Pro--poly-L-Lys, denoted (T, G)-Pro--L (10, 11). It is possible, therefore, that the genetic defect in the *low responder* DBA/1 strain could be attributed to a reduced number of immunocompetent precursor cells responsive to (T, G)-Pro--L when compared with the number in the *high-responder* SJL strain.

The limiting dilution approach has been used to estimate immunocompetent precursor cell frequencies by injecting graded and limiting numbers of spleen cells into irradiated mice, and by subsequently analyzing donor-derived responses in the recipients (16–19). The purpose of this investigation was to de-

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termine the relative numbers of such immunocompetent precursors in spleen cell suspensions of primed and normal high responder and low responder mouse strains by the limiting dilution method. Results of the present study indicate that spleen cell suspensions from immunized SJL donors contain about 25 times as many detectable precursors as suspensions from immunized DBA/1 donors. In cell suspensions from normal spleens about 4 times more immunocompetent progenitors were detected in the SJL than in the DBA/1 strain. Although preimmunization increased the number of detectable precursors in SJL donors by 5–6 times, no effect on precursor number was observed for DBA/1 spleen cells.

Materials and Methods

Mice.—Inbred animals of both sexes, 10–13 wk of age, were used as recipients, while females of the same age were used as donors. DBA/1 mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. SJL mice were obtained from the Experimental Animal Unit, the Weizmann Institute of Science, and from the Jackson Laboratory.

Immunization.—The immunogen used in this study was poly-L-(Tyr, Glu)-poly-L-Pro--poly-L-Lys, 701, denoted (T,G)-Pro--L. This is a branched synthetic polymer built from multi-poly-L-prolyl--poly-L-lysine to whose polyproline side-chains short, random peptides of tyrosine and glutamic acid are attached. Synthesis and characterization of this immunogen have been described elsewhere (20). Preimmunized spleen cell donors were injected intradermally in the hind footpads (according to standard procedure [10]) with 10 μg of (T, G)-Pro--L in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) 3 wk before cell transfer. Recipient mice received a mixture of spleen cells from the preimmunized donors and 10μg of (T, G)-Pro--L intravenously in minimal essential medium of Eagle (Grand Island Biological Co., Grand Island, N. Y.). Recipients injected with normal spleen cells were immunized intraperitoneally with 10 μg of (T, G)-Pro--L in adjuvant, 1 day after cell transfer, to ensure exposure of the transferred immunocompetent cells to the immunogen.

Irradiation.—Prospective recipient mice were exposed to 750–850 R of whole body X-irradiation (250 kv peak, 15 ma, with 0.5 mm of Cu and 1.0 mm Al, source to target distance 50 cm, and exposure rate of 60 R/min) in a rotating lucite cage.

Cell Suspensions and Transplantation.—Spleen cells were harvested as previously described (18). Nucleated cell counts were made by repeated sample counts using a hemocytometer. Injection of spleen cells into irradiated syngeneic recipients was performed as described elsewhere (18).

Passive Microhemagglutination Assay.—Sheep erythrocytes were formalinized, tanned, and coated with antigen as reported (21). Hemagglutination tests were performed on disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) by 2-fold serial dilutions of antisera in phosphate-buffered saline (0.15 N NaCl, 0.01 M phosphate buffer, pH 7) containing 0.1% bovine plasma albumin (crystallized, Armour Pharmaceutical Co., Chicago, Ill.). The plates were incubated at 20°C and read at 2.5 hr and overnight.

Statistical Methods.—The Poisson model was used to describe the theoretical probability that a known inoculum of donor-derived spleen cells would produce a significant detectable amount of anti-(T, G)-Pro--L serum in recipients (18). The method of maximum likelihood as described by Porter and Berry (22) was used to estimate the probability values and 95% confidence intervals.

RESULTS

Anti-(T,G)-Pro--L Responses in Control Mice.—In order to verify that (T, G)-Pro--L was administered in immunogenic form and that the irradiation was

sufficient to prevent host-derived response, each experiment was accompanied by two types of controls. One group of intact (nonirradiated) mice was primed at the time of donor immunization, and boosted 3 wk later at the time of immunized spleen cell transfer. Some of the above-mentioned animals were bled 12–14 days after priming and their sera were tested. As shown in Table I, DBA/1 mice responded to a single injection of (T,G)-Pro--L, although the titers were lower than those of SJL mice. Much larger differences were detected between DBA/1 and SJL mice after a second injection of antigen. Another group of animals was irradiated and injected only with (T,G)-Pro--L in adjuvant. No antibody was detected in 23 of 24 irradiated controls; a titer of 1:4 was detected in one animal. These results demonstrate that irradiation of the recipient animals was sufficient to prevent host-derived responses. The sera of normal non-immunized mice were occasionally found to show antibody titers of 1:2–1:4. For these reasons only sera of irradiated recipients (injected with spleen cells and antigen) which reacted at a dilution of 1:8 or higher were considered to contain a sufficient amount of antibody to be counted as positive.

TABLE I

Anti-(T,G)-Pro--L Titers in Control Animals 12–14 Days after Immunization

Treatment of mice	No. of sera with detectable antibody and range of hemagglutination titers	
	SJL	DBA/1
Intact mice immunized only once	5/5	5/5
	1:32	1:16
Intact mice immunized and boosted	10/10	10/10
	1:64–1:256	1:8–1:16
Irradiated mice*immunized only once	1/6	0/18
	0–1:4	0

* Exposed to 750–850 R and given antigen without spleen cells.

Frequency of Responses in Syngeneic Receipients Injected with Graded Numbers of Immunized SJL and DBA/1 Spleen Cells.—Results of previous studies indicated that there is a significant difference between the level of the secondary anti-(T,G)-Pro--L response of SJL high responder and DBA/1 low responder mouse strains (10, 11). Therefore, it was of interest in this study to estimate first the frequencies of immunocompetent precursor cells reactive with (T,G)-Pro--L in spleens of immunized SJL and DBA/1 donors.

In repeated experiments 124 SJL and 78 DBA/1 irradiated recipient mice were injected with graded numbers (1.2×10^5–6.0×10^7) of spleen cells from immunized syngeneic donors. Antigen dissolved in Eagle's medium was injected simultaneously. Recipients were bled from the retro-orbital sinus at 2–3 day intervals between the 7th and 21st days after cell transfer. The greatest number of positive donor-derived responses in recipients and the highest anti-(T,G)-

Pro--L titers were obtained between days 11 and 13 in both mouse strains. This indicates that negative sera in DBA/1 recipients cannot be attributed to a de-

TABLE II

Percentage of Positive Sera in Irradiated Syngeneic Recipients 11–13 Days after Injection of (T,G)-Pro--L and Graded Numbers of SJL or DBA/1 Spleen Cells from Immunized Donors

Strain of Mice	No. of spleen cells transplanted	Fraction of positive sera in recipients	Percentage of positive sera in recipients	Probability of positive sera per 10^6 cells*	Precursor cell frequency
	$\times 10^6$				$\times 10^{-6}$
SJL	0.12	5/18	27.7		
	0.25	7/19	36.8		
	0.50	4/15	26.7		
	1.0	11/18	61.1	0.79	1/1.26
	2.0	10/13	77.0	(0.55–0.89)‡	(1/1.12–1/1.82)‡
	4.0	16/18	89.0		
	10.0	14/15	93.4		
	50.0	8/8	100.0		
DBA/1	1.0	0/12	0		
	10.0	6/13	46.2		
	20.0	7/13	53.7	0.051	1/30
	40.0	18/25	72.0	(0.039–0.072)‡	(1/22–1/46)‡
	60.0	15/15	100.0		

* Calculated according to reference 22.

‡ 95% confidence intervals shown in parentheses.

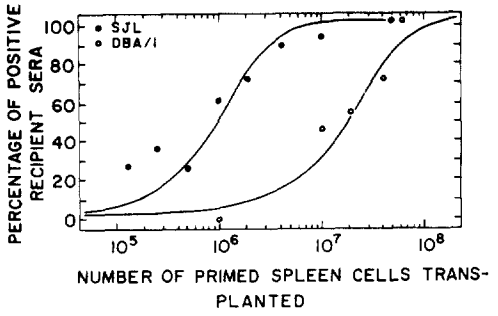

FIG. 1. Percentage of recipient mice with significant anti-(T,G)-Pro--L titers after injection of irradiated SJL and DBA/1 mice with graded numbers of immunized syngeneic spleen cells and (T, G)-Pro--L.

lay in anti-(T,G)-Pro--L response in the low responder strain. As the number of spleen cells injected increased, the proportion of recipients with positive sera also increased for both strains (Table II, Fig. 1).

Approximately 60% of SJL sera were found to be positive when 1×10^6 spleen cells were injected. However, 54–72% of DBA/1 sera were positive only after the injection of $2\text{–}4 \times 10^7$ spleen cells. The inoculum size required for

TABLE III

Percentage of Positive Sera in Irradiated Syngeneic Recipients 12–16 Days after Injection of (T,G)-Pro--L and Graded Numbers of SJL or DBA/1 Spleen Cells from Nonimmunized Donors

Strain of Mice	No. of spleen cells transplanted	Fraction of positive sera in recipients	Percentage of positive sera in recipients	Probability of positive sera per 10^6 cells*	Precursor cell frequency
	$\times 10^6$				$\times 10^{-6}$
SJL	0.5	0/9	0		
	1.0	2/9	22.1		
	4.0	6/12	50.0	0.14	1/7.15
	10.0	7/11	63.6	(0.085–0.21)‡	(1/4.76–1/11.8)‡
	20.0	10/10	100.0		
DBA/1	5.0	1/4	25.0		
	10.0	9/27	33.3		
	20.0	6/12	50.0	0.032	1/31.3
	40.0	15/25	60.0	(0.022–0.045)‡	(1/22.2–1/45.5)‡
	60.0	10/10	100.0		

* Calculated according to reference 22.

‡ 95% confidence intervals shown in parentheses.

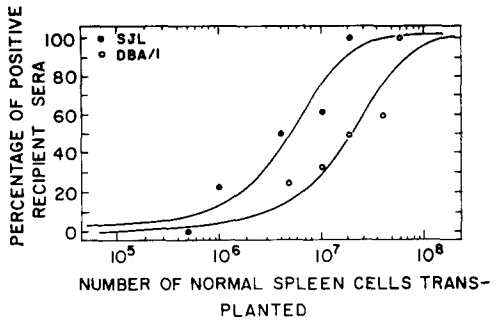

FIG. 2. Percentage of recipient mice with significant anti-(T, G)-Pro--L titers after injection of irradiated SJL and DBA/1 mice with graded numbers of nonimmunized syngeneic spleen cells and (T, G)-Pro--L.

two-thirds of the recipient sera to be positive appeared to be 20 to 40 times greater for DBA/1 than for SJL immunized spleen cells (Table II). For a more precise evaluation, the probability values that an inoculum of 10^6 spleen cells would produce a positive anti-(T,G)-Pro--L response after transfer were calcu-

lated for both mouse strains using the Poisson model (22). The value for immunized SJL spleen cells was 24 times greater than that for DBA/1 cells. The difference in these probability values was statistically significant at the 0.05 level. The curves relating inoculum size to the expected frequency of positive sera in the recipients are shown in Fig. 1, together with the observed frequencies. One (T, G)-Pro--L sensitive precursor was detected in approximately 1.3×10^6 SJL (high responder) spleen cells, whereas one relevant precursor was found in about 30×10^6 DBA/1 (low responder) spleen cells.

Frequency of Responses in Syngeneic Recipients Injected with Graded Numbers of Normal SJL and DBA/1 Spleen Cells.—Since a large difference was detected between the precursor frequencies of spleen cells from SJL and DBA/1 mice, which were previously immunized with the synthetic antigen, it was of interest to establish whether a similar difference could be observed when spleen cell suspensions from normal (nonimmunized) high and low responder mouse strains were compared. Graded and limiting numbers of SJL and DBA/1 spleen cells ranging from 5×10^5 to 6×10^7 were injected into a total of 129 irradiated syngeneic hosts. One day later the recipients were injected intraperitoneally with antigen prepared in adjuvant. Sera were tested for antibody 12, 14, and 16 days after immunization. The limiting dilution results shown in Table III and Fig. 2 indicate a significant difference in the detected precursor frequencies for spleen cells from nonimmunized donors. Approximately 4.5 times as many antigen-sensitive precursors were detected in spleens of nonimmunized SJL as in spleens of nonimmunized DBA/1 mice. The probability values were statistically significant at the 0.05 level. The detected frequencies of precursor cells for SJL and DBA/1 spleens were about 1 in 7×10^6 and 1 in 31×10^6, respectively.

DISCUSSION

Previous studies of the genetic control of immune responses indicate that it is possible to transfer responsiveness to well-defined immunogens by transferring high responder spleen cells, fetal liver cells, or peripheral blood lymphocytes into irradiated low responder animals. This has been interpreted to suggest that the genes controlling these immune responses act on a cell type directly involved in the process of antibody formation (1). However, the above-mentioned studies have not established which cell type is responsible for the genetic deficiency nor the nature of this defect. The limiting dilution assay provides a method for approaching the elucidation of these problems. In the past, limiting dilution studies have been used successfully for estimating the relative number of precursor cells for heterologous erythrocyte antigens (16–19). The results presented here are the first to demonstrate that this approach can be applied equally well for studying the cellular basis of responses to synthetic polypeptide antigens.

The experiments described indicate that there is a 24-fold greater number

of anti-(T,G)-Pro--L precursor cells in spleens of immunized SJL mice, known to be high responders, than in those of immunized DBA/1 donors, which are low responders. This confirms results obtained with intact animals, indicating that the genetic defect is quantitative (references 10 and 11, and Table I), and demonstrates that the low response of DBA/1 can be attributed to a striking reduction in the number of detected precursor cells. Furthermore, a significant 4.5-fold difference was observed in the number of splenic antigen-sensitive precursors form nonimmunized donors, suggesting that the defect is expressed even before immunization. The differences observed cannot be attributed to different temporal patterns of antibody production because the highest titers were obtained between days 11 and 13 in both mouse strains. The contrast between high and low responder animals was more pronounced when they were compared by the criterion of the number of antigen-sensitive precursor cells than by the criterion of the amount of antibodies produced in the intact mice (references 10 and 11, and Table I). The differences found between SJL and DBA/1 precursor frequencies for normal and immunized spleen cells were 4.5-fold and 24-fold, respectively, whereas the differences in titers of intact mice were only 2-fold and 8- to 16-fold. It might thus be difficult to find a significant difference between the antibody titers of genetically different strains of mice after a single injection of a synthetic antigen (23). Since genetic controls of some immune responses are expressed by quantitative differences, we have chosen to refer to SJL and DBA/1 as *high responder* and *low responder* strains, respectively, rather than the previous nomenclature of "responder" and "nonresponder" used in genetic studies of this type (1–14).

The limiting dilution experiments give estimates only of the number of *detected* precursors. It could thus be argued that the genetic defect may involve differences in affinity of precursor cells, and does not necessarily indicate differences in total numbers of relevant cells. Should this be the case, these results would still point to significant differences in the frequencies of "high affinity precursors" in high and low responder mouse strains.

A more thorough understanding of the molecular and cellular basis of immunological processes will require a detailed systematic investigation of the complex events associated with genetic control of immunity to well-defined synthetic antigens. For certain natural antigens formation of specific antibodies has been shown to require two and perhaps even three distinct cell types (24–31). The roles of these various cells are unknown, but they include recognition of antigenic determinants and production of antibodies. Functionally, the cooperating cells can be integrated into an antigen-sensitive unit (32). Genetic controls of such a complex series of events could be expressed at any of these and/or other levels. For example, separate gene loci have been found to regulate immune responses of mice to (Phe,G)-Pro--L (11). Control of the response to the (Phe,G) portion of the molecule is linked to H-2, whereas that of the re-

sponse to the (Pro--L) part is not. The same population of precursor cells in SJL mice appears to respond to (Pro--L) independently of whether the immunogen is (T, G)-Pro--L or (Phe, G)-Pro--L, and suggests that separate cell populations are reactive with the (Phe, G) and (Pro--L) portions of the molecule (Mozes, Shearer, and Sela, unpublished data).

The spleen contains a very heterogeneous population of cells, and the experiments described in the present report were not designed to establish in which functional cell type the genetic defect is expressed. Unpublished results (Shearer, Mozes, and Sela) indicate that cooperation between thymus-derived and marrow-derived cells is necessary for the anti-(T, G)-Pro--L response in mice. Therefore, it will be possible, by thymus-marrow cell transfer experiments, to determine in which immunocompetent cell population genetic control to this immunogen is expressed.

SUMMARY

SJL mice are high responders to the synthetic multichain polypeptide antigen (T, G)-Pro--L, whereas DBA/1 mice are low responders (10, 11). In order to determine whether the genetic control of immune response can be correlated with the number of antigen-sensitive precursor cells, spleen cell suspensions from normal and immunized SJL and DBA/1 donor mice were transplanted into lethally X-irradiated syngeneic recipients (incapable of immune response) along with (T, G)-Pro--L. Anti-(T, G)-Pro--L responses (donor-derived) were assayed in the sera of the hosts 12–16 days later. By transplanting graded and limiting numbers of spleen cells, inocula were found which contained one or a few antigen-sensitive precursors reactive with the immunogen. Using this method to estimate the relative numbers of such cells for the high responder SJL strain, one precursor was detected in $\sim 1.3 \times 10^6$ and $\sim 7.2 \times 10^6$ spleen cells from immunized and normal donors, respectively. In contrast, one precursor was detected in about 30×10^6 spleen cells from low responder DBA/1 mice, irrespective of whether the donors had been immunized.

These results indicate that the genetic control of immunity to the synthetic polypeptide antigen investigated is directly correlated to the relative number of precursor cells reactive with the immunogen in high and low responder strains.

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