CONTRIBUTION OF BONE MARROW CELLS AND LACK OF EXPRESSION OF THYMOCYTES IN GENETIC CONTROLS OF IMMUNE RESPONSES FOR TWO IMMUNOPOTENT REGIONS WITHIN POLY-(PHE,GLU)-POLY-PRO--POLY-LYS IN INBRED MOUSE STRAINS*

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Our knowledge of the cellular aspects of immune phenomena has been increased in recent years by the realization that cooperation between functionally distinct populations of immunocompetent cells appears to be a prerequisite for specific antibody production (1–6). The most extensively studied case of this type of cell-to-cell interaction has been exemplified in the mouse by transfer experiments with suspensions of thymus-derived and bone marrow-derived cells for immune reponses to sheep erythrocyte antigens (1, 3, 4). Similar thymus–marrow cooperation has been recently demonstrated in this species for other immunogens, including albumins (7, 8), immunoglobulins G (9), other heterologous erythrocytes (10, 11), and the branched chain synthetic polypeptide poly-L-(Tyr,Glu)-poly-L-Pro--poly-L-Lys (12).

The genetic control of immunological responsiveness has been shown to be due to a defect at the immunocompetent cell level, since irradiated low responder animals generated immune responses characteristic of high responders after injection of spleen, thoracic duct, peripheral blood, or fetal liver cells from high responder donors (13–17). In order to understand the mechanism by which genetic regulation of immunological responsiveness functions, it is necessary to determine in which immunocompetent cell population the genetic defect is expressed. Previous transfer experiments with mice have not established the cellular origin of this genetic control.

Genetic control of responses specific for two distinct immunocompetent regions within the synthetic polypeptide poly-L-(Phe,Glu)-poly-L-Pro--poly-L-Lys, denoted (Phe,G)-Pro--L, has been reported (18, 19). SJL mice were high responders to the Pro--L portion of this immunogen, but low responders to (Phe,G), whereas DBA/1 mice were high responders to the (Phe,G) determinant, but low responders to Pro--L. In two preceding publications, it was demonstrated by limiting dilution experiments that expression of such genetic control appeared to be due to insufficient numbers of relevant immunocompetent splenic precursors stimulated by the immunogens (20, 21).

In experiments reported here, cooperation between thymocytes and bone marrow cells was demonstrated for immune responses to (Phe,G)-Pro--L by cell

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transfers in mice. By limiting dilution experiments in which a nonlimiting number of thymocytes was mixed with several limiting inocula of marrow cells and transferred into syngeneic, irradiated recipients, it was found that the genetic defect of the immune response to (Phe,G) in SJL mice and to Pro--L in the DBA/1 strain could be accounted for by differences in the relative number of stimulated immunocompetent cells found in the bone marrow of these two mouse strains. In contrast, experiments involving the transfer of graded numbers of thymocytes with an excess of marrow cells indicated that the genetic control of antibody responses to (Phe,G)-Pro--L in SJL and DBA/1 mice cannot be attributed to a deficiency in the relative numbers of available thymocytes. This study also shows by means of two-step limiting dilutions that the genetic defect is not due to differences in the proliferative capacity of relevant thymocytes after stimulation with immunogen. Finally, the results indicate that a single population of thymocytes and of thymus-derived cells is triggered by (Phe,G)-Pro--L, and that these cells do not distinguish between the two immunopotent areas, namely (Phe,G) and Pro--L.

Materials and Methods

Mice.—Inbred SJL and DBA/1 mice of both sexes, 9–11 wk of age, were used as recipients, and females 8–10 wk old were used as donors of thymus and bone marrow cells. Both mouse strains were obtained from the Experimental Animal Unit, The Weizmann Institute of Science.

Irradiation.—Recipient mice to be injected with thymocytes and/or bone marrow cells were exposed to 700–800 R of whole body X-irradiation (250 kv peak, 15 ma, with 0.5 mm Cu and 1.0 mm Al, source to target distance 50 cm, and exposure rate of 60 R/min) in a rotating lucite chamber.

Cell Suspensions and Transfers.—Suspensions of bone marrow cells were prepared by flushing marrow from the femurs and tibiae of donors with cold Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.). Clumps of cells were dispersed by aspiration through a 23 gauge needle. Particulate matter was removed by filtration through 200 mesh per inch stainless steel screen.

Thymocyte suspensions were prepared by mincing the thymuses with scissors in cold Eagle's medium. The cells in the fragments were dispersed by repeated aspiration through the orifice of a 2.5 ml syringe. The cells were filtered through a stainless steel screen. Cell suspensions containing thymus-derived cells were prepared from the spleens of irradiated recipients which had been injected 1 wk earlier with syngeneic thymocytes (22, 23). These suspensions were then diluted and mixed with syngeneic marrow for injection so that a 1 ml inoculum would contain 2×10^7 marrow cells and $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, or $\frac{1}{64}$ of a spleen equivalent containing thymus-derived cells.

The concentration of each cell preparation was estimated by repeated sample counting of nucleated cells using a hemocytometer. Suspensions of thymocytes and/or marrow cells were injected into the lateral tail vein of recipients in 1 ml volumes within 4 hr after irradiation. The recipients were injected intraperitoneally with 50 USP units of heparin a few minutes before cell inoculation to prevent embolism.

Immunization.—The immunogen used in this study was poly-L-(Phe,Glu)-poly-L-Pro--poly-L-Lys, 702, abbreviated (Phe,G)-Pro--L. The synthesis and characterization of this immunogen have been previously described (24). 24 hr after irradiation, recipients of thymus

and/or bone marrow cells were given a single intraperitoneal injection of 10 μg of (Phe,G)-Pro--L in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). For experiments involving retransfers of thymus-derived cells, both the primary recipients injected with thymocytes and the secondary recipients injected with thymus-derived and marrow cells were immunized intraperitoneally with 10 μg (Phe,G)-Pro--L in complete Freund's adjuvant 24 hr after cell transfer.

Passive Microhemagglutination Assay.—The passive microhemagglutination tests performed have been described elsewhere (21), using the synthetic polypeptides poly-L-(Tyr, Glu)-poly-L-Pro--poly-L-Lys, 701, (T,G)-Pro--L (24), and poly-L-(Phe,Glu)-poly-DL-Ala--poly-L-Lys, 223, (Phe,G)-A--L (25). Separate hemagglutination assays were performed on the sera from individual recipients 14 days after immunization (the time of maximum antibody titers) using (Phe,G)-A--L– and (T,G)-Pro--L–coated erythrocytes, respectively, for detecting responses to the (Phe,G) and Pro--L regions of (Phe,G)-Pro--L.

Experimental Design and Statistical Methods.—Limiting dilution experiments were performed to estimate the frequencies of relevant immunocompetent precursors in thymus and bone marrow cell suspensions. Since an inoculum containing both thymus and marrow cells is required (under the experimental conditions used here) for the production of anti-(Phe,G) and anti-Pro--L responses (see below), graded numbers of bone marrow cells were mixed with a constant, nonlimiting number of 10^8 thymocytes. Conversely, limiting inocula of thymocytes were mixed with an excess of 20×10^6 marrow cells. These cell dilutions were made to critically reduce the frequency of one relevant cell type in the recipients, while providing an excess of the other cell (26, 27). Limiting dilution experiments for thymus-derived cells were performed by a two-step procedure similar to that reported for sheep erythrocyte antigens (23). Irradiated recipient mice were injected with 3.5×10^7 thymocytes followed by (Phe,G)-Pro--L 24 hr later. 6 days after thymocyte injection, pooled cell suspensions (containing thymus-derived cells) were made from the primary recipient spleens. Graded fractions of this suspension ranging from $\frac{1}{4}$ to $\frac{1}{64}$ of a spleen equivalent were mixed with 2×10^7 marrow cells and retransplanted into irradiated, test recipients, followed by (Phe,G)-Pro--L 24 hr later. Under the conditions outlined above, whether or not a functional immunocompetent response unit will be formed depends mainly on the number of limiting cells transferred. The Poisson model and the method of maximum likelihood were adopted for the Golem computer at the Weizmann Institute of Science and used to estimate the probability values and confidence intervals (28). The hypothesis that (Phe,G)-Pro--L stimulated a single population of thymocytes and thymus-derived cells relevant for generating anti-(Phe,G) and anti-Pro--L responses was tested by subjecting the limiting dilution results to the Yates' corrected chi-square test for association of responses assayed with (Phe,G)-A--L or (T,G)-Pro--L.

RESULTS

Thymus-Bone Marrow Cell Cooperation for (Phe,G)-Pro--L Responses.—In order to investigate the possible contribution of thymus and/or marrow cells in expression of the genetic control of immune responses to (Phe,G)-Pro--L, it was first necessary to demonstrate that cell-to-cell interaction is required for (Phe,G)– and Pro--L–specific antibody responses. Heavily irradiated SJL and DBA/1 mice were injected with thymocytes, or bone marrow cells, or with thymus and marrow cells taken from syngeneic donors, and immunized with (Phe,G)-Pro--L 24 hr later. 2 wk after cell transfer, at the time of peak antibody titers, the recipients were bled and their sera were individually assayed for (Phe,G)– and Pro--L–specific antibodies. The data obtained using the SJL

and DBA/1 strains were pooled (since the cell cooperatin results were similar) and are shown in Fig. 1. Most of the recipients injected with either thymus or marrow cells did not generate responses in the sera detectable with erythrocytes coated either with (Phe,G)-A--L or with (T,G)-Pro--L at dilutions of 1:4. Of

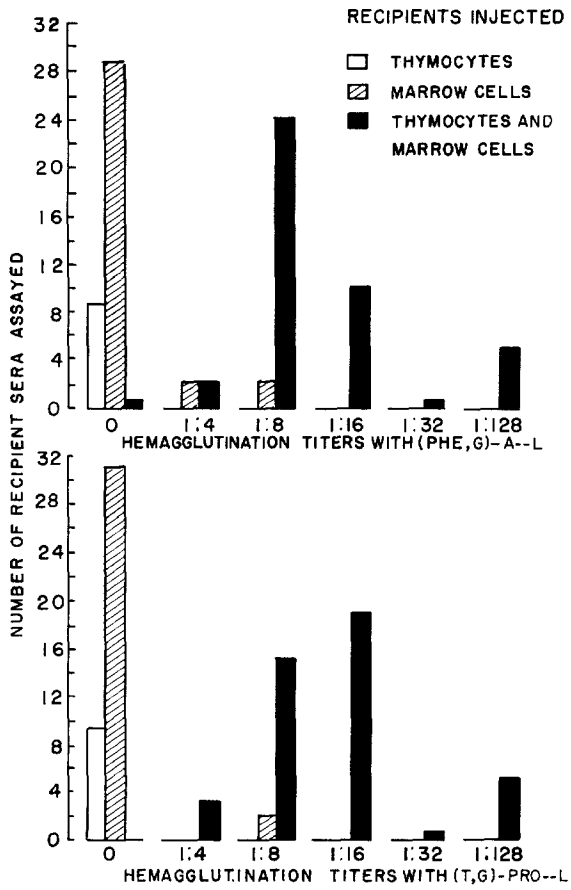

FIG. 1. Hemagglutination titers of SJL and DBA/1 recipient sera after irradiation and injection of (Phe,G)-Pro--L and 10^8 thymocytes (symbol □), 20×10^6 marrow cells (symbol ▨), or 100×10^6 thymocytes and 20×10^6 marrow cells (symbol ■) from syngeneic donors. The sera were assayed with (Phe,G)-A--L or (T,G)-Pro--L. The vertical axis plots the number of recipient sera showing a given hemagglutination titer.

the 16 SJL mice injected with bone marrow cells, only one gave a detectable response to Pro--L. 2 of the 17 DBA/1 marrow recipients responded to (Phe,G) with titers of 1:8 and 1 of 17 gave a similar response to Pro--L. None of the 9 DBA/1 mice injected with thymocytes generated detectable antibody responses for the two specificities. In contrast, most recipients

from both mouse strains inoculated with a mixture containing 60–100 $\times$ 10^6 thymocytes and 20 $\times$ 10^6 marrow cells generated responses specific for (Phe,G) and Pro--L at serum dilutions greater than 1:4. Of the 21 SJL recipients injected with the cell mixtures, 13 gave hemagglutination titers with (Phe,G)-A--L at a dilution of 1:8, 2 at 1:16, and 5 at 1:128. From the same sera assayed with (T,G)-Pro--L, 6 were positive at 1:8, 10 at 1:16, and 5 at 1:128. From a total of 22 DBA/1 thymus–marrow recipients, 11 gave hemagglutination at 1:8, 8 at 1:16, and 1 at 1:32, when the sera were tested with (Phe,G)-A--L. The same sera titered with (T,G)-Pro--L resulted in 9 mice showing a titer at 1:8, 9 at 1:16, and 1 at 1:32. These results indicate that thymocytes and marrow cells interact synergistically to generate (Phe,G)– and Pro--L–specific responses in both mouse strains.

Frequency of Responses in SJL and DBA/1 Recipients Injected with 10^8 Thymocytes and Graded Numbers of Marrow Cells.—Expression of the genetic control of antibody responses to (Phe,G)-Pro--L (21) and to a related branched chain, synthetic polypeptide, (T,G)-Pro--L (20), has been correlated with differences in the relative numbers of limiting precursor cells detected in the spleens of inbred mouse strains. Suspensions of immunocompetent cells from other lymphoid sources which express this immune defect should also exhibit differences in the frequencies of the relevant precursors in high and low responder mice. On the other hand, limiting dilutions of lymphoid cells not expressing the defect would be expected to generate about equally frequent responses in both high and low responder animals. Since both thymocytes and marrow cells are required to generate antibodies specific for (Phe,G) and for Pro--L (see Fig. 1), limiting dilutions of one of these cell populations in the presence of a constant excess number of the other cell provides a convenient approach for investigating the contribution of thymus and/or bone marrow in expression of the genetic control of immunity.

In the present study, irradiated SJL and DBA/1 mice were injected with graded inocula of marrow cells (0.05–20 $\times$ 10^6) mixed with 10^8 thymocytes prepared from syngeneic donors. The recipients were injected with 10 μg of (Phe,G)-Pro--L in complete Freund's adjuvant 1 day later. 2 wk after cell transfer, at the time of peak antibody titer, the recipients were bled and their sera assayed for (Phe,G)– and Pro--L–specific antibodies using (Phe,G)-A--L– and (T, G)-Pro--L–coated erythrocytes, respectively. The sera were classified as "positive" only if antibodies specific for (Phe,G) or Pro--L were detected at dilutions greater than 1:4 (reference 21 and Fig. 1). The results of 106 SJL and 107 DBA/1 surviving recipients are shown in Tables I and II and in Figs. 2 and 3. Similar to the results obtained with limiting inocula of spleen cells from unimmunized syngeneic donors (21), differences of about 5-fold were observed in the frequencies of (Phe,G)– and Pro--L–specific responses in both strains of mice. For the SJL strain (low responder to [Phe,G]), an inoculum of 1 $\times$ 10^6 marrow cells was sufficient to generate Pro--L responses in two-thirds of the recipients,

whereas the transfer of more than 8×10^6 cells was required to generate (Phe,G) responses in an equivalent proportion of mice (Table I). Conversely, 1×10^6 syngeneic marrow cells generated positive (Phe,G) responses in two-thirds of the DBA/1 recipients (low responders to Pro--L), whereas 8×10^6 transferred cells were required to yield a similar frequency of sera positive

TABLE I

Percentage of Positive Sera in Irradiated SJL Recipients 14 Days After Injection of (Phe,G)-Pro--L, 10^8 Thymocytes, and Graded Numbers of Bone Marrow Cells from Syngeneic Donors

Sera of recipients titered with	No. of marrow cells transplanted ($\times 10^6$)	Fraction of positive sera in recipients*	Percentage of positive sera in recipients*	Probability of positive sera per 10^6 cells‡	Precursor cell frequency ($\times 10^{-6}$)
(Phe,G)-A--L	0.05	0/9	0		
	0.1	0/5	0		
	0.25	0/16	0		
	0.5	1/7	14.3		
	1.0	6/21	28.6	0.14	1/7.3
	2.0	6/20	30.0	(0.094–0.20)§	(1/5.0–1/11)§
	4.0	4/11	36.4		
	8.0	9/21	42.8		
	20.0	6/6	100.0		
(T,G)-Pro--L	0.05	1/9	11.1		
	0.1	1/5	20.0		
	0.25	8/16	50.0		
	0.5	4/7	57.1		
	1.0	14/21	66.7	0.57	1/1.7
	2.0	14/20	70.0	(0.41–0.80)§	(1/1.2–1/2.4)§
	4.0	9/11	81.8		
	8.0	18/21	85.6		
	20.0	6/6	100.0		

* Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.

‡ Estimates of probability values were based on the Poisson model (28).

§ 95% confidence intervals shown in parentheses.

for Pro--L (Table II). For a more accurate evaluation of the frequency data which considers all inocula tested, the probability values that 10^6 marrow cells (in the presence of a nonlimiting number of thymocytes) would generate detectable (Phe,G) or Pro--L responses were estimated using the Poisson model (28). These probability values for SJL marrow donors were 0.14 and 0.57 for (Phe,G) and Pro--L, respectively, and the corresponding frequencies for limiting marrow precursors were $\frac{1}{7.3} \times 10^6$ and $\frac{1}{1.7} \times 10^6$ (Table I, Fig. 2). The

95% confidence intervals did not overlap for the responses assayed with (Phe,G)-A--L. The probabilities that 10^6 DBA/1 marrow cells would contain precursor cells relevant for generating (Phe,G) or Pro--L responses were 0.53 and 0.15, respectively, and the precursor frequencies were estimated to be $\frac{1}{1.9} \times 10^6$ for (Phe,G) and $\frac{1}{6.5} \times 10^6$ for Pro--L (Table II, Fig. 3). These differences were significant at the 0.05 level. The frequency data shown here in-

TABLE II

Percentage of Positive Sera in Irradiated DBA/1 Recipients 14 Days After Injection of (Phe,G)-Pro--L, 10^8 Thymocytes, and Graded Numbers of Bone Marrow Cells from Syngeneic Donors

Sera of recipients titered with	No. of marrow cells transplanted ($\times 10^6$)	Fraction of positive sera in recipients*	Percentage of positive sera in recipients*	Probability of positive sera per 10^6 cells‡	Precursor cell frequency ($\times 10^{-6}$)
(Phe,G)-A--L	0.1	2/11	18.2		
	0.25	3/13	23.1		
	0.5	10/26	38.5		
	1.0	5/8	62.5	0.53	1/1.9
	2.0	8/11	73.0	(0.37–0.75)§	(1/1.3–1/2.7)§
	4.0	14/17	82.4		
	8.0	9/11	81.8		
	20.0	10/10	100.0		
(T,G)-Pro--L	0.1	0/11	0		
	0.25	0/13	0		
	0.5	3/26	11.5		
	1.0	3/8	37.5	0.15	1/6.5
	2.0	3/11	27.3	(0.10–0.23)§	(1/4.4–1/9.7)§
	4.0	8/17	47.1		
	8.0	7/11	63.6		
	20.0	9/10	90.0		

* Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.

‡ Estimates of probability values were based on the Poisson model (28).

§ 95% confidence intervals shown in parentheses.

dicate that the low responsiveness of SJL mice to (Phe,G) can be accounted for by a 5-fold reduced number of detected marrow precursors when compared with marrow precursors generating the Pro--L response. Conversely, the 5-fold lower number of immunocompetent marrow cells generating Pro--L responses, compared with those generating (Phe,G) responses in DBA/1 mice, is correlated with the fact that the DBA/1 strain is a poor responder to Pro--L.

Frequency of Responses in SJL and DBA/1 Recipients Injected with 2×10^7 Marrow Cells and Graded Numbers of Thymocytes.—In order to estimate the

relative numbers of thymus cells stimulated by (Phe,G)-Pro--L, graded inocula of syngeneic thymocytes (0.5–100 $\times$ 10^6) were mixed with 2 $\times$ 10^7 bone marrow cells and injected into groups of heavily irradiated SJL and DBA/1 mice. The recipients were immunized with (Phe,G)-Pro--L and their sera as-

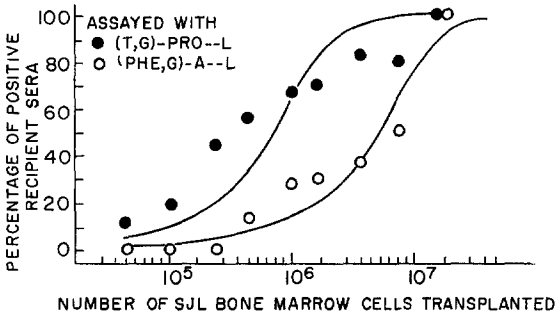

FIG. 2. Percentage of positive sera in SJL recipients when assayed with (Phe,G)-A--L (symbol ○) or (T,G)-Pro--L (symbol ●) after irradiation and injection of (Phe,G)-Pro--L, 10^8 thymocytes, and graded numbers of bone marrow cells from syngeneic donors.

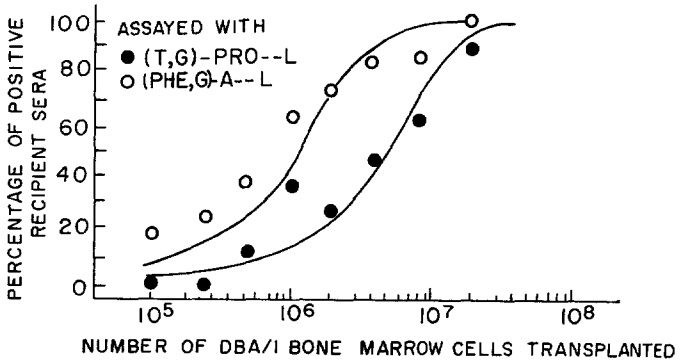

FIG. 3. Percentage of positive sera in DBA/1 recipients when assayed with (Phe,G)-A--L (symbol ○) or (T,G)-Pro--L (symbol ●) after irradiation and injection of (Phe,G)-Pro--L, 10^8 thymocytes, and graded numbers of bone marrow cells from syngeneic donors.

sayed for (Phe,G) and Pro--L responses as described above. The results obtained from 116 SJL and 116 DBA/1 survivors are summarized in Tables III and IV and in Figs. 4 and 5. In contrast to the results obtained with limiting numbers of transferred spleen (21) or bone marrow cells (see above), no significant differences were detected when the responses were limited by the numbers of thymocytes transferred for either specificity in these two mouse strains. A transfer of 10–20 $\times$ 10^6 SJL thymocytes was required to generate (Phe,G) or

Pro--L responses in two-thirds of the recipients. The probability values that 10^6 SJL thymocytes would contain immunocompetent cells that generated positive (Phe,G) or Pro--L responses was calculated to be 0.089 and 0.10, respectively. The corresponding precursor frequencies were estimated to be $\frac{1}{11} \times 10^6$ and $\frac{1}{10} \times 10^6$ for these two specificities, and the 95% confidence intervals over-

TABLE III

Percentage of Positive Sera in Irradiated SJL Recipients 14 Days After Injection of (Phe,G)-Pro--L, 2 × 10^7 Bone Marrow Cells, and Graded Numbers of Thymocytes from Syngeneic Donors

Sera of recipients titered with	No. of thymocytes transplanted ($\times 10^6$)	Fraction of positive sera in recipients*	Percentage of positive sera in recipients*	Probability of positive sera per 10^6 cells‡	Precursor cell frequency ($\times 10^{-6}$)
(Phe,G)-A--L	0.5	1/6	16.7		
	1.0	7/21	33.3		
	2.5	7/21	33.3		
	5.0	7/13	53.8	0.089	1/11
	10.0	10/18	55.5	(0.065–0.12)§	(1/8.1–1/15)§
	20.0	10/13	77.0		
	40.0	12/14	85.7		
	60.0	9/10	90.0		
(T,G)-Pro--L	0.5	1/6	16.7		
	1.0	7/21	33.3		
	2.5	9/21	42.9		
	5.0	8/13	61.5	0.10	1/10
	10.0	10/18	55.5	(0.074–0.14)§	(1/7.1–1/14)§
	20.0	10/13	77.0		
	40.0	14/14	100.0		
	60.0	10/10	100.0		

* Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.
‡ Estimates of probability values were based on the Poisson model (28).
§ 95% confidence intervals shown in parentheses.

lapped (Table III and Fig. 4). In order to obtain an equivalent proportion of positive sera in the DBA/1 strain, about 20×10^6 thymocytes had to be injected. The calculated probabilities were 0.051 and 0.053 and the corresponding frequencies were $\frac{1}{20} \times 10^6$ and $\frac{1}{19} \times 10^6$ for (Phe,G) and Pro-L responses (Table IV and Fig. 5). No differences were found between the (Phe,G) and Pro--L thymocyte precursor frequencies within the mouse strains, and the 1.9-fold frequency differences observed between the strains were not significant. The relationship between the number of thymocytes injected and the proportion of positive recipients generally followed the predictions of the Poisson model, al-

though for the DBA/1 strain some divergence from the expected frequencies was observed at the lower inocula. These variations could have been due to fluctuations in the numbers of thymocytes injected in repetitive experiments.

Frequency of Responses in SJL and DBA/1 Recipients Injected with 2 × 10^7 Marrow Cells and Dilutions of Thymus-Derived Cells in a Two-Step Experiment.

TABLE IV

Percentage of Positive Sera in Irradiated DBA/1 Recipients 14 Days After Injection of (Phe,G)-Pro--L, 2 × 10^7 Bone Marrow Cells, and Graded Numbers of Thymocytes from Syngeneic Donors

Sera of recipients titered with	No. of thymocytes transplanted (×10^6)	Fraction of positive sera in recipients*	Percentage of positive sera in recipients*	Probability of positive sera per 10^6 cells‡	Precursor cell frequency (×10^{-6})
(Phe,G)-A--L	0.5	1/9	11.1		
	1.0	5/18	27.7		
	2.5	2/6	33.3		
	5.0	10/21	47.6		
	10.0	9/17	53.0	0.051	1/20
	20.0	7/11	63.6	(0.036–0.073)§	(1/14–1/28)§
	40.0	8/12	66.7		
	60.0	10/12	83.3		
	100.0	10/10	100.0		
(T,G)-Pro--L	0.5	1/9	11.1		
	1.0	4/18	22.2		
	2.5	2/6	33.3		
	5.0	10/21	47.6		
	10.0	9/17	53.0	0.053	1/19
	20.0	7/11	63.6	(0.037–0.074)§	(1/14–1/27)§
	40.0	8/12	66.7		
	60.0	10/12	83.3		
	100.0	9/10	90.0		

* Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.

‡ Estimates of probability values were based on the Poisson model (28).

§ 95% confidence intervals shown in parentheses.

The possibility has to be considered that the genetic defect might not be due to differences in the initial numbers of potentially responding thymocytes, but that after cell transfer and immunization, thymocytes specific for the high-responding determinant could proliferate more rapidly than those specific for the low-responding determinant. Were this to be the case, a greater number of thymus-derived descendent cells specific for Pro--L would be expected than the number specific for (Phe,G) in SJL mice. Conversely, a higher frequency of (Phe,G)-

specific than Pro--L–specific thymus-derived cells would be expected in DBA/1 mice. The proliferative capacity of thymocytes stimulated by sheep erythrocytes has been estimated by a two-step limiting dilution assay (23). We have used a similar approach for (Phe,G)-Pro--L by injecting irradiated mice with

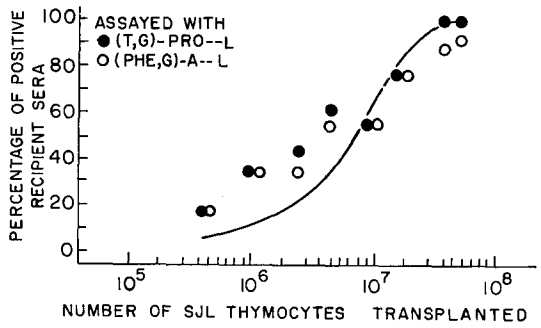

FIG. 4. Percentage of positive sera in SJL recipients when assayed with (Phe,G)-A--L (symbol ○) or (T,G)-Pro--L (symbol ●) after irradiation and injection of (Phe,G)-Pro--L, 2×10^7 bone marrow cells, and graded numbers of thymocytes from syngeneic donors.

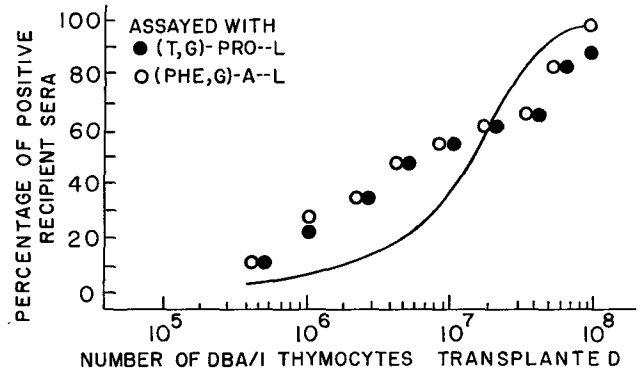

FIG. 5. Percentage of positive sera in DBA/1 recipients when assayed with (Phe,G)-A--L (symbol ○) or (T,G)-Pro--L (symbol ●) after irradiation and injection of (Phe,G)-Pro--L, 2×10^7 bone marrow cells, and graded numbers of thymocytes from syngeneic donors.

35×10^6 syngeneic thymocytes and (Phe,G)-Pro--L. For both mouse strains an inoculum of this size would be expected to contain an average of 2–4 detectable thymic precursors (see Tables III and IV). 7 days after thymocyte transfer, during which time the relevant thymus population proliferated (22, 23, 29), recipient spleens containing thymus-derived cells were removed, pooled, and suspensions made. These cell suspensions were serially diluted, mixed with 2×10^7 syngeneic bone marrow cells, and injected into a second group of test

recipients with (Phe,G)-Pro--L. Sera from the test recipients were individually assayed 12 days later, and the results are summarized in Table V and Fig. 6. No differences were detected by dilution of thymus-derived cells in either strain for the (Phe,G)– or Pro--L–specific responses. Sera from about two-thirds of the SJL and DBA/1 test recipients were positive for both specificities when $\frac{1}{8}$ of a spleen equivalent containing thymus-derived cells was transferred with an ex-

TABLE V

*Percentage of Positive Sera in Irradiated SJL and DBA/1 Test Recipients After Injection of (Phe,G)-Pro--L, 2 × 10^7 Syngeneic Marrow Cells, and Graded Fractions of Spleens Containing Thymus-Derived Cells**

Fraction of primary spleen retransplanted with marrow	Recipient sera titered with (T,G)-Pro--L			Recipient sera titered with (Phe,G)-A--L		
	Fraction of positive sera	Percentage of positive sera	Probability of positive sera‡	Fraction of positive sera	Percentage of positive sera	Probability of positive sera‡
			SJL strain			
1/16	7/13	53.8		7/13	53.8	
1/8	8/13	61.5	0.11	8/13	61.5	0.11
1/4	5/7	71.4	(0.071–0.18)§	5/7	71.4	(0.071–0.18)§
			DBA/1 strain			
1/64	2/9	22.2		2/9	22.2	
1/32	3/9	33.3		3/9	33.3	
1/16	7/14	50.0	0.076	7/14	50.0	0.076
1/8	10/15	66.7	(0.051–0.11)§	10/15	66.7	(0.051–0.11)§
1/4	9/14	64.4		9/14	64.4	

* Primary recipients were injected with 35 × 10^6 thymocytes and 10 μg of (Phe,G)-Pro--L 24 hr later. Fractions of thymus-derived cells that lodged and proliferated in spleens were retransplanted into test recipients 7 days later with marrow cells. Test recipients were immunized with 10 μg of (Phe,G)-Pro--L 24 hr later.

‡ Probability of obtaining a positive test recipient sera per 1/100 of retransplanted primary spleen containing thymus-derived cells.

§ 95% confidence limits shown in parentheses.

cess of marrow, irrespective of whether the mouse strain under study was a high or a low responder to the determinants assayed. Statistical treatment of the data yielded identical probability values for the two specificities within each strain (Table V), and slight but insignificant differences (0.11 for SJL and 0.076 for DBA/1) in the probabilities between the strains.

Chi-Square Analysis of (Phe,G)- and Pro--L–Specific Responses from Thymocyte and Thymus-Derived Cell Limiting Dilutions.—In order to determine whether the equally frequent dilutions of (Phe,G) and Pro--L responses were the result of two separate but superimposed curves (one responsible for generating (Phe,G)–specific and the other Pro--L–specific responses), or whether the responses to both determinants were the result of a single curve (indicating a

common thymic precursor cell for both specificities), the frequency data from individual recipients were subjected to a chi-square test for independence of

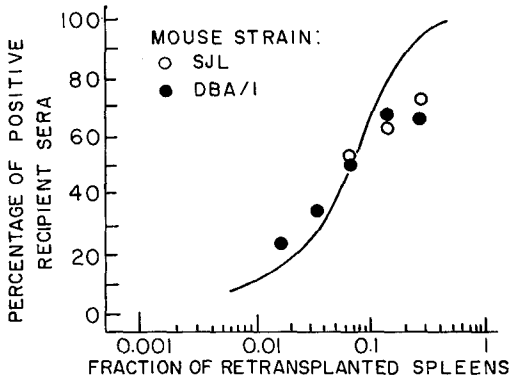

FIG. 6. Percentage of positive sera in SJL (symbol ○) and DBA/1 (symbol ●) test recipients when assayed with (Phe,G)-A--L or (T,G)-Pro--L after irradiation and injection of (Phe,G)-Pro--L, 2×10^7 bone marrow cells, and fractions of repopulated spleens from irradiated primary recipients which had been injected with 35×10^6 thymocytes and (Phe,G)-Pro--L 1 wk earlier. All cell transfers were made in syngeneic recipients.

TABLE VI

Chi-Square Tests for Independence of (Phe,G)- and Pro--L-Specific Responses in Recipients of 20 × 10^6 Marrow Cells and Graded Numbers of Thymocytes or Thymus-Derived Cells, Immunized with (Phe,G)-Pro--L

Response detected in recipient sera titered with		No. of sera tested for thymocytes in		No. of sera tested for thymus-derived cells in	
(T,G)-Pro--L	(Phe,G)-A--L	SJL	DBA/1	SJL	DBA/1
+	+	38	33	18	28
+	−	6	6	2	3
−	+	12	5	2	3
−	−	26	43	14	27
Total number of sera compared*		82	87	36	61
Chi-square values‡		21.3	44.9	19.0	36.5

* Results of only those inocula which generated appreciable numbers of positive and negative recipient sera were used in the chi-square tests.

‡ Yates' corrected chi-square values in the table were compared with 10.8, the critical value of χ^2 statistic at the 0.001 level of significance.

(Phe,G) and Pro--L responses. Only results of those inocula in which appreciable numbers of positive and negative sera were found (i.e. 1–40 × 10^6 thymocytes) were used in this statistical test, so as not to bias the data in favor of association of responses. The results, summarized in Table VI, including the calculated chi-square values, indicate that there was association (i.e. lack of in-

dependence) between the generation of (Phe,G)- and Pro--L–specific antibody responses, when these responses were limited by transferring critical numbers of either thymocytes or thymus-derived cells. For the SJL strains, the calculated chi-square values were 21.3 and 19.0 for thymocytes and thymus-derived cells, respectively. For DBA/1 mice, these values were calculated to be 44.9 for thymocytes and 36.5 for thymus-derived cells. These calculated values are well above 10.8, the critical value of chi-square at the 0.001 level of significance. All the comparisons are compatible with the hypothesis that responses to (Phe,G) and Pro--L are not independent of each other when the thymus-derived population of cells limits the response to (Phe,G)-Pro--L.

DISCUSSION

Evidence has been previously presented that expression of the genetic regulation of immune responses of inbred mouse strains to synthetic polypeptides built on multichain poly-L-proline can be correlated with the relative numbers of antigen-sensitive units stimulated in cell transfer experiments involving spleen cells (20, 21). Since mouse spleens contain a mixture of immunocompetent cells of thymus and bone marrow origin (30–32), it was necessary to first establish thymus–marrow cell cooperation for (Phe,G)-Pro--L responses before investigation of the cellular source of the genetic defect could be performed. The observation that thymus and marrow cells were needed for immune responses to (Phe,G)-Pro--L is in agreement with the work of Tyan et al. (33) which demonstrated that thymectomized, irradiated, fetal liver-protected mice did not respond to a branched chain synthetic polypeptide. Furthermore, it adds a synthetic immunogen to the growing list of natural antigens which require cell-to-cell interaction for immune responses (1–11).

Since both thymus and marrow cells were necessary for the generation of anti-(Phe,G) and anti-Pro--L responses, cell suspensions from either of these organs which carry the defect might also be expected to show differences in precursor frequencies in high and low responder mice. Such differences should be demonstrable by limiting dilution transfer in irradiated syngeneic recipients. The frequencies of immunocompetent precursors in the marrow of SJL and DBA/1 mice generating (Phe,G)- and Pro--L–specific responses were similar to results reported for spleen cell suspensions for unimmunized donors of these two strains (21). About five times more precursors were detected for Pro--L than for (Phe,G) in the marrow of SJL donors (high responders to Pro--L). Conversely, in the DBA/1 high responder strain to (Phe,G), a 5-fold greater number of marrow precursors was observed that generated responses specific for (Phe,G) than for Pro--L. In contrast to the direct correlation obtained between the strain-dependent immune response potentials and the relative numbers of immunocompentent precursors detected in bone marrow cells, no differences were observed in the frequencies of thymocytes relevant for

generating (Phe,G) and Pro--L responses. For each mouse strain the confidence intervals overlapped for the two specificities, and the 1.9-fold estimated frequency differences between the two mouse strains were not significant at the 0.05 level. Therefore, although immunological responsiveness to (Phe,G)-Pro--L is genetically controlled in these two mouse strains, the differential expression of ability to respond was not seen when generation of (Phe,G)– and Pro--L–specific antibodies was dependent on a critical number of available thymocytes. The main finding of this study is, thus, that the expression of the genetic control, which had previously been correlated with a reduced number of low responder mouse spleen cells (20, 21), was also found to be a characteristic of bone marrow cells but not of thymocytes. The experimental design used in this report provides an approach for investigating the cellular basis of genetic regulation of immune responsiveness in syngeneic animals, and therefore avoids graft–host rejection problems which could interfere with interpretation of the results.

The use of a defined, synthetic immunogen (which possesses a restricted number of antigenic determinants), in conjunction with the limiting dilution cell transfer in high and low responder mouse strains, provides an opportunity for investigating the specificity restriction of immunocompetent cells. The fact that many recipients given critical inocula of SJL marrow cells (with an excess of thymus cells) exhibited responses positive for Pro--L but negative for (Phe,G), and that most irradiated mice injected with limiting numbers of DBA/1 marrow (with a nonlimiting number of thymocytes) produced (Phe,G)–specific but not Pro--L–specific responses, indicated that the least frequent cell type relevant for generating responses to these polypeptide determinants in mouse bone marrow is committed for a single antigenic specificity. A similar conclusion has been reached for the limiting cell type reactive with (Phe,G)-Pro--L in SJL, DBA/1, and F_1 spleens (21). The above findings are compatible with recent observations of others that immunocompetent cells in bone marrow can be specifically rendered tolerant (9) or specifically immunized (10, 11, 34), despite the fact that these marrow elements cooperate with "antigen-reactive cells" of thymus origin in order to elicit antibody production (3). The fact that thymocyte dilution experiments resulted in equally frequent responses for (Phe,G) and Pro--L in the two mouse strains could mean that there were two populations of thymocytes, one specific for (Phe,G) and the other for Pro--L, both of which were present in equal frequencies and stimulated by (Phe,G)-Pro--L. Alternatively, a single population of thymus cells could have been triggered by the immunogen which generated, via cooperation with monospecific marrow cells, two distinct populations of antibodies, one specific for (Phe,G), and the other specific for Pro--L (19). Results of chi-square analysis of the limiting dilution data are compatible only with the second of these possibilities (see Table VI), since the transfer of the response to (Phe,G) was almost always accompanied by the transfer of the response to Pro--L in thymocyte di-

lution experiments. The observation that (Phe, G) and Pro--L immune responses were associated in transfer experiments involving limiting numbers of thymocytes and an excess of bone marrow cells could be accounted for by one of the following possibilities: (*a*) that stimulation of a single population of thymocytes specific for only one of these determinants is sufficient to generate specific antibody production to both (Phe, G) and Pro--L; or (*b*) that the population of thymocytes relevant for generating (Phe, G) and Pro--L responses is specific for the intact (Phe, G)-Pro--L immunogenic macromolecule. From the experiments reported here, it is not possible to distinguish between these two mechanisms. However, if the thymocyte population were specific for only one of these immunopotent areas, but could generate specific antibodies for both regions via marrow cells, then one would have expected to detect (Phe, G) responses in recipients which did not generate Pro--L responses, and vice versa. Thus, the strong association of (Phe, G) and Pro--L responses favors the second mechanism, i.e., that a single population of thymocytes is specific for the whole polypeptide macromolecule. Differences in receptor specificity for immunocompetent cells necessary for cell-mediated immunity and antibody-forming cell precursors have been recently reported (35). Furthermore, polyvalent specificity has been demonstrated for lymphocytes involved in cytotoxic reactions with allogeneic target cells (36, 37). This latter finding contrasts with the observations reported here that marrow cells appear to be restricted to generate either (Phe, G)– or Pro--L–specific antibodies, and raises the possibility that bone marrow precursors of antibody-synthesizing cells are more restricted in their antigen reactivity than are thymocytes. Irrespective of whether thymus cells "recognize" all of the immunogen or only a part of it, the strong association between (Phe, G)– and Pro--L–specific antibody production, when the immune system was limited by thymocytes, implies that a single population of such cells is activated by (Phe, G)-Pro--L.

It is interesting that the frequency of thymocytes relevant for generating (Phe, G)-Pro--L responses (1 per 1–2 $\times$ 10^7 thymus cells) in SJL and DBA/1 mice is similar to the frequency values obtained in other mouse strains for thymic antigen-reactive cells responsive to sheep erythrocyte antigens (23, 27). If a complex immunogen such as sheep erythrocytes (SRBC) can be assumed to possess more antigenic determinants than the branched chain synthetic polypeptides (38, 39), then one would expect to detect a higher total frequency of thymocytes for SRBC than for (Phe, G)-Pro--L, providing that a separate population of thymus cells exists for each determinant. If, on the other hand, the thymus-derived population of cells does not distinguish between individual determinants irrespective of the total number of determinants present, then about equally frequent populations of thymocytes would be expected to be stimulated by either a complex or a simple immunogen, as was demonstrated.

The possibility exists that expression of the genetic defect could reside at the

level of the thymus-derived cell, and that this defect would not necessarily be detected by transplanting graded numbers of thymocytes. For example, if the low responses were to result from different rates of proliferation of equal numbers of preexisting thymic antigen-reactive cells in the two mouse strains, then no differences would be detected in the initial numbers of responding thymocytes. The differences in the proliferative capacities of these cells would then become apparent only some time after they had been triggered by the immunogen. The identical frequencies obtained for (Phe,G) and Pro--L responses by dilution of thymus-derived cells from spleens of irradiated recipients 1 wk after injection of 35×10^6 thymocytes and (Phe,G)-Pro--L indicate that the low response of SJL to (Phe,G) and of DBA/1 to Pro--L cannot be attributed to differences in the rates of proliferation of (Phe,G)- or Pro--L–specific thymus-derived precursors. Furthermore, the dependence of (Phe-G) and Pro--L responses for thymus-derived cells in both mouse strains (Table VI) indicates that the descendents of thymic antigen-reactive cells do not distinguish between (Phe,G) and Pro--L. In other words, a single population of thymus-derived cells generates distinct (Phe,G)- and Pro--L–specific responses, probably via interaction with separate monospecific populations of marrow precursors. From these results it can be concluded that the thymus population of immunocompetent cells does not acquire greater restriction with respect to antigenic specificity after exposure to immunogen. The equal frequencies of thymocytes and thymus-derived cells obtained for (Phe,G) and Pro--L within the two mouse strains, and the strong association observed for these responses, makes it difficult to visualize how genetic regulation of responses for two immunopotent regions within the same macromolecule could be attributed to a cell population which itself does not distinguish between these two immunopotent areas. Nevertheless, these experiments do not necessarily exclude other cellular aspects which might relate genetic control of responsiveness to thymocytes.

Cells contained in bone marrow have been found to be restricted for antibody class (26), allotype (40), and determinant specificity (this study). Marrow-derived elements are also known to be the precursors of plaque-forming cells (31), and appear to contain at least one population of rosette-forming cells (11). It is not known whether all of the above-mentioned functions are characteristic of a single marrow cell type, or whether they are distributed among subpopulations in the marrow. Genetic controls of immunity have been considered to be due to defects in the antigenic recognition and/or the antibody production phases of immune responses (16, 41). The experiments presented in this report demonstrate a genetic defect at the level of the bone marrow population(s) of immunocompetent precursors, but not at the thymocyte level. Such thymus–marrow transfer experiments, however, do not necessarily permit conclusions to be drawn regarding these so-called "regcognition" or "production" steps. Although thymocytes appear to be antigen-specific (3, 7, 9, 22, 23), and do not generate

antibody-forming cells (42, 43), whereas marrow elements are the precursors of plaque-forming cells (31), it cannot be excluded that bone marrow cells also play an active role in recognition. In fact, results of recent studies indicate the presence of specific precursors in bone marrow (9–11, 34). Since marrow contains cells possessing the properties of recognition and of antibody production, it is not possible from the data presented here to determine whether the immunological genetic defect for (Phe,G)-Pro--L is one of antigenic recognition, of antibody production, or of both. Evidence has been presented suggesting a three cell antigen-sensitive unit (2,4); two of these cells appear to be marrow-derived and independent of the thymus (4). Thus, it is possible either that one marrow cell is concerned with recognition and the other with antibody production, or that a single marrow cell type functions both for recognition and for antibody production. Should the latter possibility be correct and the genetic control of (Phe,G)-Pro--L responses be associated exclusively with marrow, then it would not be possible to distinguish defects involving recognition from those involving production.

SUMMARY

Previous cellular studies on the genetic regulation of immunological responsiveness for two immunopotent regions within the branched chain synthetic polypeptide (Phe,G)-Pro--L demonstrated a direct correlation between the number of detectable immunocompetent splenic precursor cells and the response patterns of SJL, DBA/1, and F_1 mice (21). In order to establish the cellular origin(s) of the genetic defect, the present study first demonstrated that thymus and bone marrow cell cooperation was required for (Phe,G)– and Pro--L–specific immune responses. Secondly, limiting dilution experiments, in which several graded and limiting inocula of marrow cells were mixed with a non-limiting number of 10^8 thymocytes and injected into irradiated, syngeneic recipients, indicated that the low responsiveness of the SJL and DBA/1 strains to the (Phe,G) and Pro--L specificities, respectively, could be attributed to a reduced number of precursor cells found in bone marrow. About five times more marrow precursors were detected in SJL mice for Pro--L than for (Phe,G), whereas about five times as many precursor cells were estimated for (Phe, G) as for Pro--L in the DBA/1 strain. These differences are similar to those obtained using spleen cells from unimmunized SJL and DBA/1 donors (21), and indicate that these genetically determined variations in responsiveness can be accounted for by differences in the frequencies of monospecific populations of immunocompetent cells present in bone marrow.

In contrast, limiting dilution transfers of thymocytes or thymus-derived cells with an excess of syngeneic marrow cells resulted in equally frequent (Phe,G) and Pro--L responses for both SJL ad DBA/1 strains. This finding in conjunction with the observation that the generation of (Phe,G)– and Pro--L–specific

responses were associated in individual recipients injected with limiting inocula of thymocytes indicated that a single population of thymocytes was stimulated by (Phe,G)-Pro--L. Therefore, it is improbable that the thymic population of immunocompetent cells contributes to expression of these genetically controlled defects.

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