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

II. FREQUENCY OF IMMUNOCOMPETENT PRECURSORS SPECIFIC FOR TWO DISTINCT REGIONS WITHIN (Phe, G)-Pro--L, A SYNTHETIC POLYPEPTIDE DERIVED FROM MULTICHAIN POLYPYROLINE, IN INBRED MOUSE STRAINS*

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Studies with synthetic polypeptide antigens of limited heterogeneity have contributed to the realization that genetics plays a role in the regulation of immune responsiveness (1–6). Such genetic control has a cellular basis, since immune responses of mice to synthetic polypeptides derived from multichain polyalanine and responses of guinea pigs to conjugates of poly-L-lysine have been obtained by repopulating the immune system of nonresponder animals with lymphoid cells from responders (1, 7, 8). Furthermore, phenotypic expression of this type of genetic control of immunity to the synthetic polypeptide poly-L-(Tyr, Glu)-poly-L-Pro--poly-L-Lys, denoted (T, G)-Pro--L, was recently shown to be reflected in the relative numbers of immunocompetent units of response detected in the spleens of high and low responder mouse strains (9). About 24 times more limiting precursor cells were detected for this immunogen in spleens of immunized high responder SJL than in those of low responder DBA/1 mice. Therefore, the cellular manifestation of genetic regulation of immunological responsiveness to (T, G)-Pro--L appears to be due to insufficient numbers of relevant precursor cells stimulated by the immunogen under the experimental conditions used.

Mozes, et al. reported genetic control of antibody specificity for the synthetic polypeptide poly-L-(Phe, Glu)-poly-L-Pro--poly-L-Lys, abbreviated (Phe, G)-Pro--L (6). DBA/1 mice responded well to the (Phe, G) determinant, but poorly to the Pro--L portion of this polypeptide. Conversely, the SJL strain was found to be a high responder to Pro--L, but a low responder to (Phe, G). This synthetic immunogen offers particular advantages for studying a number of fundamental immunological problems and their possible relationship to the genetic control of immunological

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responsiveness. For example, since the two immunopotent portions of the (Phe, G)-Pro--L macromolecule appear to be under separate genetic regulation (6), one can systematically investigate the cellular manifestations of this type of genetic control in relation to problems such as the recognition of the immunogen, the commitment of immunocompetent precursor cells for a given specificity, the nature of the lymphoid organs exhibiting the phenotypic expression of the defect, and the possible significance of histocompatibility loci in the development of the immune state. Although some of the above immunological questions have been studied using heterologous erythrocytes (10-13), the large number of determinants contributed by such antigens makes an already complex series of events even more difficult to comprehend.

In the present study, the limiting dilution approach (9, 14) was used to estimate the least frequent cell type which constitutes an antigen-sensitive unit responsible for generating immune responses specific for (Phe, G) and for Pro--L in SJL, DBA/1, and F1 mice. With respect to the basic immunological problems outlined above, the findings of this report show that: limiting dilution analysis can be used for studying the cellular basis of responsiveness to different immunopotent areas within the same macromolecule; different populations of spleen cells limit the responsiveness to (Phe, G) and Pro--L; and genetic control of responsiveness to different determinants attached to poly-L-proline can dramatically alter the secondary response to the Pro--L itself.

Materials and Methods

Mice.—Inbred SJL and DBA/1 mice and F1 animals of both sexes, 9-11 wk of age, were used as recipients, whereas females of the same age were used as donors. Both inbred mouse strains were obtained from the Jackson Laboratories, Bar Harbor, Maine, and from the Experimental Animal Unit, The Weizmann Institute of Science. The F1 hybrids of a cross between SJL and DBA/1 parents were raised in our department.

Immunization.—The immunogen used in this work was poly-L-(Phe, Glu)-poly-L-Pro--poly-L-Lys, abbreviated (Phe, G)-Pro--L. The description, synthesis, and characterization of this immunogen have been previously described (15). Immunization of donor and recipient mice with (Phe, G)-Pro--L was performed as reported elsewhere (9).

Irradiation and Cell Transfers.—Recipient mice to be injected with spleen cells were exposed to 700-800 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 autoclave chamber. Spleen cell suspensions were prepared and injected into irradiated syngeneic recipients as described elsewhere (9, 14). Nucleated cell counts were made by repeated sample counting using a hemocytometer.

Microhemagglutination Assay.—The synthetic polypeptides used for assay were poly-L-(Try, Glu)-poly-L-Pro--poly-L-Lys, 701, (T, G)-Pro--L (15), and poly-z-(Phe, Glu)-poly-oz-Ala--poly-z-Lys, 223, (Phe, G)-A--L (16). Sheep erythrocytes were formalinized, tanned, and coated either with (T, G)-Pro--L or with (Phe, G)-A--L, as previously described (17). Passive microhemagglutination tests (18) were performed on disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) by 2-fold serial dilutions of antisera in phosphate-buffered saline (0.15 x 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. Separate hemagglutination assays were performed using (T, G)-Pro--L-coated and (Phe, G)-A--L-coated erythrocytes on the sera from individual recipient mice 12-14 days after immunization, i.e., at the time of maximum antibody titers (9).

Statistical Methods.—The Poisson model was used to describe the theoretical probability that a given inoculum of donor-derived spleen cells would generate a significant detectable amount of anti-(Phe, G) or anti-Pro--L serum in the irradiated recipients (14). The maximum likelihood method was used to estimate the probability values and 95% confidence intervals (19).

Frequency of Responses in Syngeneic Recipients Injected with Graded Numbers of Spleen Cells from Immunized SJL and DBA/1 Donors.—Results of earlier studies in which mice were immunized with (Phe, G)-Pro--L indicated that significant differences existed between the levels of secondary anti-(Phe, G) and anti-Pro--L responses in SJL and DBA/1 mice (5, 6). SJL mice were found to be high responders to Pro--L, but low responders to the (Phe, G) determinant of (Phe, G)-Pro--L. Conversely, DBA/1 animals were high responders to (Phe, G), but low responders to the Pro--L portion of the immunogen. In cell transfer experiments, a 24-fold difference in the frequency of limiting precursor cells relevant for the (T, G)-Pro--L response was detected in spleens of immunized SJL and DBA/1 donors, whereas a less dramatic but significant difference of 4-5-fold was observed using spleen cells from nonimmunized donors of these two strains (9). Therefore, in the present work, limiting dilution analyses were first performed using spleen cell suspensions from immunized SJL and DBA/1 donors in order to detect the maximum possible frequency differences of precursors limiting responsiveness to (Phe, G) and Pro--L.

In repeated experiments a total of 116 SJL and 74 DBA/1 irradiated recipient mice were injected with graded numbers (1 X 10^5 — 4 X 10^5) of spleen cells pooled from immunized syngeneic donors. Each prospective donor had been immunized intradermally 3 wk before cell transfer with 10 μg of (Phe, G)-Pro--L in complete Freund's adjuvant (9). Each recipient animal received an intravenous injection of spleen cells pooled from immunized donors mixed with 10 μg of (Phe, G)-Pro--L dissolved in Eagle's medium. The recipients were bled from the retro-orbital plexus at the time of peak antibody responses, i.e., 12 days after cell transfer (9).

The sera were individually assayed for antibodies specific for (Phe, G) and for Pro--L by titering with (Phe, G)-A--L and (T, G)-Pro--L, respectively. Sera from control animals, i.e., nonirradiated, uninjected mice or irradiated mice injected with antigen only, either exhibited no detectable antibodies or gave responses detectable at a dilution not greater than 1:4. Therefore, sera of recipient animals were considered to be positive or negative, depending on whether or not antibodies specific for (Phe, G) and/or Pro--L were detected at dilutions of serum greater than 1:4. The results are presented in Tables I and II, and summarized graphically in Figs. 1 and 2.

An increase was observed in the fraction of sera positive for (Phe, G) and
### TABLE I

**Percentage of Positive Sera in Irradiated SJL Recipients 12 Days after Injection of (Phe, G)-Pro–L and Graded Numbers of Syngeneic Spleen Cells from Immunized Donors**

| Sera of recipients titered with | Number of spleen cells transplanted (X 10⁶) | Fraction of positive sera in recipients* | Percentage of positive sera in recipients | Probability of positive sera per 10⁶ cells† | Precursor cell frequency (X 10⁻⁹) |
|---------------------------------|---------------------------------------------|-----------------------------------------|------------------------------------------|------------------------------------------|----------------------------------|
| (Phe, G)-A–L                   | 0.1 0/8                                      | 0                                       | 0                                        | 1/20                                     | (0.034 - 0.074)§ (1/13 - 1/29)§ |
|                                | 0.5 1/21                                      | 4.5                                     | 0.050                                    |                                         |                                  |
|                                | 1 2/18                                       | 11.1                                    |                                          |                                         |                                  |
|                                | 2 2/15                                       | 13.3                                    |                                          |                                         |                                  |
|                                | 5 8/15                                       | 53.3                                    |                                          |                                         |                                  |
|                                | 10 8/15                                      | 53.3                                    |                                          |                                         |                                  |
|                                | 40 11/16                                     | 68.8                                    |                                          |                                         |                                  |
| (T, G)-Pro–L                   | 0.1 1/8                                      | 12.5                                    |                                          |                                         |                                  |
|                                | 0.5 1/22                                     | 54.5                                    |                                          |                                         |                                  |
|                                | 1 14/21                                      | 77.8                                    | 0.74                                     | 1/1.3                                    |                                  |
|                                | 2 12/15                                      | 80.0                                    | (0.65 - 1.3)§                           | (1/0.77 - 1/1.5)§                       |                                  |
|                                | 5 21/22                                      | 95.5                                    |                                          |                                         |                                  |
|                                | 10 13/15                                     | 86.6                                    |                                          |                                         |                                  |
|                                | 40 16/16                                     | 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 (19).  
§ 95% confidence intervals shown in parentheses.

### TABLE II

**Percentage of Positive Sera in Irradiated DBA/1 Recipients 12 Days after Injection of (Phe, G)-Pro–L and Graded Numbers of Syngeneic Spleen Cells from Immunized Donors**

| Sera of recipients titered with | Number of spleen cells transplanted (X 10⁶) | Fraction of positive sera in recipients* | Percentage of positive sera in recipients | Probability of positive sera per 10⁶ cells† | Precursor cell frequency (X 10⁻⁹) |
|---------------------------------|---------------------------------------------|-----------------------------------------|------------------------------------------|------------------------------------------|----------------------------------|
| (Phe, G)-A–L                   | 0.1 5/7                                      | 28.6                                    |                                          |                                         |                                  |
|                                | 2 14/21                                      | 66.7                                    |                                          |                                         |                                  |
|                                | 1 10/10                                      | 80.0                                    | (0.35 - 0.87)§                           | (1/1.1 - 1/2.6)§                       |                                  |
|                                | 10 12/13                                     | 92.4                                    |                                          |                                         |                                  |
|                                | 30 16/16                                     | 100.0                                   |                                          |                                         |                                  |
| (T, G)-Pro–L                   | 0.1 3/7                                      | 14.3                                    |                                          |                                         |                                  |
|                                | 2 4/7                                       | 57.2                                    | 0.11                                     | 1/9.4                                    |                                  |
|                                | 4 4/10                                      | 80.0                                    | (0.07 - 0.16)§                           | (1/6.2 - 1/14)§                       |                                  |
|                                | 10 10/13                                     | 77.0                                    |                                          |                                         |                                  |
|                                | 30 14/16                                     | 87.5                                    |                                          |                                         |                                  |

* Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.  
† Estimates of probability values were based on the Poisson model (19).  
§ 95% confidence intervals shown in parentheses.
for Pro—L in both mouse strains as the number of spleen cells injected was increased. As shown in Table I for the Pro—L response, between 55 and 78% positive SJL sera were obtained after injection of 0.5—1 × 10⁶ spleen cells, whereas an equivalent proportion of sera were positive for the (Phe, G) response only after injection of more than 10 × 10⁶ spleen cells. The inoculum size that corresponded to significant antibodies detected in two-thirds of the recipient sera was 10–40 times greater for the (Phe, G) determinant than for Pro—L. For a more precise evaluation (which takes into account all inocula tested) the probability values that 10⁶ spleen cells would generate positive (Phe, G) or Pro—L responses were estimated using the Poisson model and the method of maximum likelihood (19). The values for spleen cells from immunized SJL donors was 15 times greater for Pro—L than for (Phe, G). The difference in these probability values was statistically significant at the 0.05 level. The curves relating the number of cells injected to the frequencies of positive sera are shown in Fig. 1. In spleens of immunized SJL mice, one limiting precursor cell relevant for the Pro—L response was detected in 1.3 × 10⁶ cells, whereas one precursor was detected in 20 × 10⁶ cells for the (Phe, G) response. It is noteworthy that the sera of all 30 animals which were positive for (Phe, G) were also positive for Pro—L. In contrast, of the 89 sera which contained significant amounts of Pro—L antibodies, 59 did not exhibit demonstrable quantities of (Phe, G) antibodies.

Table II presents the frequency data of responses for the two immunopotent portions of (Phe, G)—Pro—L in spleens of immunized DBA/1 mice. Injections of 1 × 10⁶ and 10 × 10⁶ cells, respectively, were required to generate detectable (Phe, G) and Pro—L responses in the sera of approximately two-thirds of the recipients. Statistical treatment of the data as outlined above indicated that the probability values that 10⁶ DBA/1 spleen cells would generate (Phe, G) or Pro—L responses were 0.58 and 0.11, respectively. This 5-fold difference
was significant. The limiting dilution curves for (Phe, G) and Pro---L responses in spleens of immunized DBA/1 donors are illustrated in Fig. 2. One limiting and relevant precursor for the (Phe, G) response and one for the Pro---L response were detected in 1.7 × 10⁶ and in 9.4 × 10⁶ spleen cells, respectively. Sera of 32 of the 35 mice positive for Pro---L were also detected as positive for (Phe, G), whereas the sera of 27 of the 59 mice positive for (Phe, G) were not observed to be positive for Pro---L.

Using (T, G)-A--L as a cross-reacting antigen, it was previously shown that the anti-(T, G)-Pro---L response was directed mainly against the Pro---L portion of the molecule (5). Therefore, it was of interest to establish whether Pro---L precursor frequencies would be similar when (T, G)-Pro---L or (Phe, G)-Pro---L were used as the immunogens. A comparison of the results reported earlier (9), in which the immunogen was (T, G)-Pro---L, with those presented above, in which the immunogenic macromolecule was (Phe, G)-Pro---L, points to a discrepancy in the immunocompetent potential of DBA/1 spleen cells which was not anticipated. Whereas the frequency of limiting precursors detected in spleens of immunized DBA/1 donors was previously found to be 1/30 × 10⁶ cells for (T, G)-Pro---L (9), a much higher frequency of 1/9.4 × 10⁶ spleen cells was now observed for the Pro---L portion of (Phe, G)-Pro---L (Table II). Thus, although preimmunization did not alter the relative numbers of limiting precursors for (T, G)-Pro---L in the DBA/1 strain, there may have been an effect of priming on the Pro---L response when (Phe, G)-Pro---L was used as the immunizing agent. In other words, the anti-Pro---L response in boosted animals appears to differ, depending on whether the immunogen contains (Phe, Glu) or (Tyr, Glu) attached to Pro---L. In order to investigate this possibility, additional limiting dilution studies were performed using spleen cell suspensions from nonimmunized SJL and DBA/1 donors.

**Frequency of Responses in Syngeneic Recipients Injected with Graded Numbers**
of Spleen Cells from Nonimmunized SJL and DBA/1 Donors.—In duplicate experiments, 83 SJL and 75 DBA/1 mice were irradiated and injected with graded inocula of cells \(1 \times 10^6 - 4 \times 10^7\) harvested from pooled spleens of nonimmunized syngeneic donors. 1 day later, the recipients were injected intraperitoneally with 10 \(\mu\)g (Phe, G)-Pro--L in complete Freund's adjuvant. Sera taken from individual recipients 14 days later were titered for (Phe, G)-A--L and Pro--L antibodies and classified as positive or negative, as described above. The results are shown in Tables III and IV and Figs. 3 and 4. As the number of inoculated spleen cells was increased, there was a corresponding increase in the proportion of positive recipient sera titered with (Phe, G)-A--L and (T, G)-Pro--L in both strains.

Approximately two-thirds of the sera of SJL mice were positive for Pro--L after injection of \(5 \times 10^6\) spleen cells, whereas an equivalent fraction of sera positive for (Phe, G) was detected only after injection of \(20 \times 10^6\) cells (Table...
The probability values that $10^6$ inoculated spleen cells from nonimmunized SJL donors would yield positive (Phe, G) and Pro--L responses were calculated to be 0.049 and 0.30, respectively. The corresponding limiting dilution curves are shown in Fig. 3. The frequency of splenic precursors limiting the response for (Phe, G) was estimated to be 6-fold less than that for Pro--L. Unlike the results obtained with recipients of immunized SJL spleen cells, 8 of

### TABLE III

Percentage of Positive Sera in Irradiated SJL Recipients 14 Days after Injection of (Phe, G)--Pro--L and Graded Numbers of Syngeneic Spleen Cells from Nonimmunized Donors

| Serum of recipients | Number of spleen cells transplanted ($\times 10^9$) | Fraction of positive sera in recipients† | Percentage of positive sera per $10^6$ cells‡ | Probability of positive sera per $10^6$ cells§ | Precursor cell frequency ($\times 10^{-6}$) |
|---------------------|-----------------------------------------------|--------------------------------------|---------------------------------------------|---------------------------------------------|------------------------------------------|
| (Phe, G)--Pro--L    | 1                                             | 2/10                                  | 20.0                                        | 0.049                                       | 1/20                                    |
|                     | 2                                             | 3/12                                  | 25.0                                        |                                             |                                          |
|                     | 5                                             | 6/18                                  | 33.3                                        | (0.036 - 0.068)§                            | (1/15 - 1/28)§                          |
|                     | 10                                            | 7/13                                  | 53.8                                        | (T, G)--Pro--L                             |                                          |
|                     | 20                                            | 6/10                                  | 60.0                                        |                                             |                                          |
|                     | 40                                            | 16/20                                 | 80.0                                        |                                             |                                          |
| (T, G)--Pro--L      | 1                                             | 4/10                                  | 40.0                                        | 0.30                                        | 1/3.4                                   |
|                     | 2                                             | 7/12                                  | 58.2                                        |                                             |                                          |
|                     | 5                                             | 12/18                                 | 66.7                                        | (0.20 - 0.43)§                              | (1/2.3 - 1/4.9)§                        |
|                     | 10                                            | 12/13                                 | 92.3                                        |                                             |                                          |
|                     | 20                                            | 10/10                                 | 100.0                                       |                                             |                                          |
|                     | 40                                            | 18/20                                 | 90.0                                        |                                             |                                          |

* Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.  
† Estimates of probability values based on Poisson model (19).  
§ 95% confidence intervals shown in parentheses.

the 40 sera positive for (Phe, G) did not show detectable anti-Pro--L activity in recipients of nonimmunized SJL spleen cells.

Results of experiments using spleen cells from nonimmunized DBA/1 donors are presented in Table IV and Fig. 4. Estimates of the probability values per $10^6$ transferred cells were calculated to be 0.12 and 0.026 for the (Phe, G) and Pro--L specificities, respectively. This 4.5-fold difference in limiting precursor frequency is illustrated in Fig. 4.

The number of precursors necessary to generate an anti-Pro--L response in spleens of nonimmunized DBA/1 donors was 1 in 38 million, after immunization with (Phe, G)--Pro--L. This number is not significantly different from the frequency of 1 in 30-31 million, obtained upon immunization with (T, G)--Pro--L, either after transfer from nonimmunized or from immunized donors.
Thus, the anti-Pro-L response can indeed be increased in the (Phe, G)-Pro-L system, but not in the (T, G)-Pro-L system.

18 of the 19 sera that were positive for the Pro-L specificity were also positive for (Phe, G), whereas 23 of the total 41 (Phe, G)-positive sera were negative for Pro-L.

Frequency of Responses in F1 Recipients Injected with Graded Numbers of Spleen Cells from Immunized Syngeneic Donors.—In the results described above,

| Sera of recipients titered with | Number of spleen cells transplanted (× 10^9) | Sera of recipients titered with | Number of spleen cells transplanted (× 10^9) |
|--------------------------------|---------------------------------------------|--------------------------------|---------------------------------------------|
| (Phe, G)-Pro-L                 |                                              | (T, G)-Pro-L                   |                                              |
| 0.5                            | 2/14 14.3                                   | 0.5                            | 1/14 7.1                                    |
| 1                              | 4/11 36.4                                   | 1                              | 0/11 0                                      |
| 2                              | 7/14 50.0                                   | 2                              | 1/14 7.1                                    |
| 5                              | 4/7 57.1                                    | 5                              | 2/7 28.6                                    |
| 10                             | 3/4 75.0                                    | 10                             | 1/4 25.0                                    |
| 20                             | 11/14 78.6                                  | 20                             | 7/14 50.0                                   |
| 40                             | 10/11 91.0                                  | 40                             | 7/11 63.6                                   |

* Donor-derived responses showing titers at dilutions greater than 1:4 in recipient sera.
† Estimates of probability values were based on the Poisson model (19).
§ 95% confidence intervals shown in parentheses.

a considerable proportion of DBA/1 sera were positive for (Phe, G), but negative for Pro-L, whereas a significant fraction of SJL sera were positive for Pro-L, but negative for (Phe, G). These observations raise the question of whether the relevant and limiting cell types for (Phe, G) and for Pro-L are restricted to generating immune responses of a single specificity. The responses of intact (DBA/1 X SJL) F1 mice to (Phe, G) and Pro-L were found to be intermediate between those of the two parental strains (6). Results obtained in such studies indicated that separate populations of antibody molecules were synthesized to the (Phe, G) and the Pro-L parts of the immunogen. Therefore, by taking advantage of the fact that F1 animals respond well to the two
| Sera of recipients | Number of spleen cells transplanted (X 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 (X 10^-6) |
|-------------------|---------------------------------------------|------------------------------------------|--------------------------------------------|------------------------------------------|-------------------------------|
| (Phe, G)–A--L     | 0.5                                        | 5/18                                     | 27.9                                       |                                          | 1/3.0                         |
|                   | 1                                           | 4/14                                     | 28.6                                       |                                          |                               |
|                   | 2                                           | 3/10                                     | 30.0                                       | 0.33                                     | (1/2.0 - 1/4.5)§              |
|                   | 3                                           | 6/15                                     | 40.0                                       | (0.22 - 0.49)§                           |                               |
|                   | 4                                           | 6/12                                     | 50.0                                       |                                          |                               |
|                   | 8                                           | 9/11                                     | 81.8                                       |                                          |                               |
| (T, G)–Pro--L     | 0.5                                        | 3/18                                     | 16.7                                       |                                          | 1/2.8                         |
|                   | 1                                           | 3/14                                     | 21.5                                       |                                          |                               |
|                   | 2                                           | 3/10                                     | 30.0                                       | 0.36                                     | (1/1.9 - 1/4.0)§              |
|                   | 3                                           | 10/15                                    | 66.7                                       | (0.25 - 0.53)§                           |                               |
|                   | 4                                           | 7/12                                     | 58.3                                       |                                          |                               |
|                   | 8                                           | 11/11                                    | 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 (19).
§ 95% confidence intervals shown in parentheses.

determinants of (Phe, G)–Pro--L, limiting dilution experiments and chi-square tests were performed in order to establish whether the responses to (Phe, G) and to Pro--L are independent. Irradiated F1 recipient mice were injected with (Phe, G)–Pro--L and graded numbers (0.5 – 8.0 X 10^6) of syngeneic spleen cells from immunized donors. The limiting dilution results are shown in Table V and Fig. 5. The frequencies of limiting precursors for (Phe, G) and for Pro--L were calculated to be 1/3.0 X 10^6 and 1/2.8 X 10^6, respectively. The probabil-

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**Fig. 5.** Percentage of positive sera in F1 recipients when assayed with (Phe, G)–A--L (symbol O) or (T, G)–Pro--L (symbol •) after irradiation and injection of (Phe, G)–Pro--L and graded numbers of spleen cells from immunized syngeneic donors.
ity values were not significantly different for responses to the two determinants, and the 95% confidence intervals overlapped. Since the frequency results for responses to (Phe, G) and Pro--L were indistinguishable, data from the range of cell inocula (0.5 - 4.0 × 10^6) that resulted in appreciable numbers of positive and negative recipient sera were combined and subjected to a Yates corrected chi-square test for independence of (Phe, G) and Pro--L responses. Of the 79 sera considered, 9 were positive and 28 were negative for both specificities. 15 sera showed antibody activity to (Phe, G), but not to Pro--L, whereas Pro--L specific antibodies were detected in 17 sera that did not show antibodies specific for (Phe, G). The calculated chi-square value was 0.1, well below the critical value of 3.84 at the 0.05 level of significance. This statistical result is in agreement with the notion that the splenic precursors limiting the response to (Phe, G) and Pro--L are independent.

**DISCUSSION**

The objectives of this study were: (a) to extend to another antigen the previous report (9) that phenotypic expression of genetic control to synthetic polypeptide antigens is directly correlated to the number of immunocompetent responses detected; (b) to demonstrate this relationship for two separate antigenic determinants on the same immunogen; and (c) to establish whether the responses to these two portions of the antigenic macromolecule are transferred independently.

The estimated frequency of limiting spleen cell precursors specific for Pro--L from immunized SJL donors was 1/1.3 × 10^6 (Table I, Fig. 1), similar to that found for (T, G)--Pro--L in the same strain (9). As was expected from the low response of intact SJL mice to (Phe, G) (6), the relative number of detected precursors was 1/20 × 10^6, 15 times below that of the Pro--L carried on the same immunogenic macromolecule. In contrast, the relative number of limiting precursors detected for (Phe, G) in spleens of immunized DBA/1 mice was 1/1.7 × 10^6, 12 times higher than that observed in SJL spleens (Table II, Fig. 2).

The frequency of precursors reactive with Pro--L observed in spleen cells from preimmunized DBA/1 donors was surprisingly high (1/9.4 × 10^6) when compared to that calculated for (T, G)--Pro--L (1/30 × 10^6) (9). These differences indicate that significant changes can be seen in the frequency of precursors specific for a given determinant when it is part of another immunogen. This could have been due to an initially greater number of precursors and/or to an increase in the number of precursors detected as a result of immunization. Indeed, results of additional experiments (Table IV, Fig. 4) indicate that the frequency of Pro--L precursor cells in such spleens was 1/38 × 10^6, 4-fold lower than that found after preimmunization with (Phe, G)--Pro--L, but similar to the frequency calculated for precursors in spleens of immunized or
nonimmunized DBA/1 donors when injected with (T, G)-Pro--L. Although preimmunization had no detectable effect when (T, G)-Pro--L was the immunogen, there was a significant increase in precursor frequency to Pro--L when (Phe, G)-Pro--L was used. Even though the short amino acid sequences attached to Pro--L do not determine in this case the specificity of the antibodies produced, they do influence the number of immunocompetent units responding to the Pro--L determinant.

For the (Phe, G) specificity, to which DBA/1 mice are high responders, a 5-fold increase (1/1.7 x 10^6 vs. 1/8.5 x 10^6) in frequency was observed after preimmunizing the donors, whereas preimmunization had no detectable effect on limiting (Phe, G) precursor frequency in SJL mice—low responders to (Phe, G). Preimmunization of SJL donors resulted in a slight (2.6-fold) but significant increase in the number of Pro--L specific precursors (Table III, Fig. 3).

The unequal frequencies of immunocompetent response units for the (Phe, G) and Pro--L specificities in the two parental strains raised the possibility that the limiting precursors for the two determinants may have been precommitted before immunization. The striking differences observed between limiting (Phe, G) and Pro--L precursor frequencies in SJL and DBA/1 spleens imply either that these cells were precommitted for the specificity before stimulation with immunogen, or that there was a preferential unidirectional commitment of a noncommitted cell to (Phe, G) in DBA/1 mice and to Pro--L in SJL mice at the time of immunization. If either of these possibilities were correct, then the response to the two specificities would be expected to be transferred to irradiated recipients independently. Since in the parental strains the frequencies of responses were so different, it was not possible to test the dependence between (Phe, G) and Pro--L responses. The similar frequencies for the above responses (1/3.0 x 10^6 and 1/2.8 x 10^6, respectively) obtained using F1 spleens enabled us to investigate this question (Table V, Fig. 5).

The finding that equal numbers of relevant spleen cells limiting the response to (Phe, G) and Pro--L were detected in F1 mice could mean either that a single cell type was restricting the response to the (Phe, G)-Pro--L macromolecule, or that two cells of equal frequency but of different specificities, i.e. (Phe, G) or Pro--L, were limiting the response. In other words, there could have been either a single dilution curve, which limited the response to the intact immunogen, or there could have been a superimposition of two curves, one which limited the response to (Phe, G) and the other to Pro--L. The result of the chi-square test for independence of (Phe, G) and Pro--L responses in F1 mice is compatible with the latter possibility. Therefore, it is likely that the spleen cells limiting the response in these experiments were restricted to generate antibodies specific for (Phe, G) or for Pro--L.

Since genetic control of antibody specificity has been demonstrated for
(Phe, G)–Pro–L (6), and since this report shows a direct correlation between responsiveness and limiting precursor frequency, as well as independence of (Phe, G) and Pro–L responses, it is possible to predict that phenotypic expression of the genetic defect(s) under study should be demonstrable only in lymphoid tissues which exhibit independence of responses to (Phe, G) and Pro–L. On the other hand, if a population of cells can be found which recognizes the intact (Phe, G)–Pro–L immunogen, then such a population should not reflect the observed genetic defect. Studies are in progress to investigate the above hypothesis.

SUMMARY

DBA/1 mice are high responders to the (Phe, G) determinant of the synthetic polypeptide (Phe, G)–Pro–L, whereas SJL mice respond well to the Pro–L region of this macromolecule (6). In order to determine whether the phenomenon described above is related to the number of antigen-sensitive units detected for both specificities, and whether responses to these determinants can be transferred independently, graded and limiting inocula of spleen cells from SJL, DBA/1, and F1 donors were injected into X-irradiated, syngeneic, recipient mice with (Phe, G)–Pro–L.

By this approach, one antigen-sensitive unit specific for (Phe, G) was detected in $1.7 \times 10^6$ and $8.5 \times 10^6$ spleen cells from immunized and nonimmunized DBA/1 donors, respectively. In contrast, one (Phe, G) relevant precursor was detected in $20 \times 10^6$ SJL spleen cells, irrespective of whether the donors had been immunized. On the other hand, for the Pro–L specificity, one limiting splenic precursor was found in $1.3 \times 10^6$ and in $3.4 \times 10^6$ cells for immunized and nonimmunized SJL donors, respectively; whereas one response unit was estimated for this determinant in $9.4 \times 10^6$ and in $38 \times 10^6$ spleen cells from immunized and nonimmunized DBA/1 mice. The findings reported here indicate that the phenotypic expression of the genetic control(s) for immune responsiveness to different immunopotent regions of (Phe, G)–Pro–L is directly correlated with the number of immunocompetent response units detected in two inbred mouse strains.

In the spleens of immunized F1 donors, similar frequencies of one limiting precursor in $3.0 \times 10^6$ and in $2.8 \times 10^6$ cells were detected for (Phe, G) and Pro–L, respectively. The results of a chi-square test for independence of (Phe, G) and Pro–L responses in F1 animals is compatible with the hypothesis that the transferred spleen cells limiting the response to (Phe, G)–Pro–L are restricted to generate antibodies specific for only one of the two determinants of this macromolecule.

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