

## ANTIGEN STRUCTURAL REQUIREMENTS FOR IMMUNOGLOBULIN ISOTYPE SWITCHING IN MICE\*

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Structurally simple synthetic molecules have proven useful for elucidating relationships between the structure and immunologic properties of antigens (1, 2) because it is possible to chemically modify such molecules to address specific questions. Although immunogens are generally considered to be large molecules—as opposed to haptens—and the most potent clearly are, a number of noteworthy exceptions to this principle have been recorded. For example, L-tyrosine-*p*-azobenzene-*p*-arsonate (RAT),<sup>1</sup> with a molecular weight of only ~400 daltons, induces in guinea pigs specific delayed hypersensitivity, DNA synthesis by lymphocytes from sensitized animals, and functional carrier activity for anti-hapten responses (2). However, the murine anti-hapten PFC response to the bifunctional antigen used in guinea pigs, *N*-2,4-dinitrophenyl-6-amino-caproyl-tyrosine-*p*-aminobenzene-*p*-arsonate (DNP-SAC-RAT), was extremely weak (2). Evidence is presented here that the response to this antigen in mice is exclusively of the IgM class in both primary and secondary responses.

It is generally believed that IgM antibody responses are less T dependent than IgG responses, and that carrier-specific helper T cells are responsible for the switch from IgM to other Ig isotypes (3). Indeed, it has been recently suggested that at least two types of carrier-specific helper cells synergize for optimal antibody responses, one of which is Ig-dependent (4).

In view of these emerging concepts, a possible basis for the weak, exclusively IgM anti-2,4-dinitrophenyl (DNP) plaque-forming cell (PFC) response in mice to DNP-SAC-RAT could be its monovalency with respect to carrier epitopes, which might limit interaction to a single helper T cell. This consideration prompted an investigation of the effect of adding a second homologous carrier epitope to the bifunctional conjugate, creating, in effect, a trifunctional antigen comprised of two identical carrier epitopes and a single haptenic epitope. The murine response to this trifunctional antigen is the major theme of this report.

\* Aided by grant AI 05664 from the U. S. Public Health Service.

<sup>1</sup> *Abbreviations used in this paper:* ABA, *p*-azobenzene-*p*-arsonate; Ac-PRO<sub>10</sub>-RAT, acetyl-(proline)<sub>10</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate; BI-1 [or DNP-SAC-RAT], *N*-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-*p*-azobenzene-*p*-arsonate; BI-2 [or DNP-SAC-PRO<sub>9</sub>-RAT], *N*-2,4-dinitrophenyl-6-amino-caproyl-(proline)<sub>9</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate; BI-3 [or DNP-SAC-PRO<sub>10</sub>-RAT], *N*-2,4-dinitrophenyl-6-amino-caproyl-(proline)<sub>10</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate; CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenyl; DNP-SAC-PRO<sub>9</sub>-TYR, *N*-2,4-dinitrophenyl-6-amino-caproyl-(proline)<sub>9</sub>-tyrosine; GM, geometric mean(s); HBSS, Hanks' balanced salt solution; KLH, keyhole limpet hemocyanin; MEM, minimum essential medium, PBS, phosphate-buffered saline; PFC, plaque-forming cell(s); PPS, PFC per spleen; RAT, L-tyrosine-*p*-azobenzene-*p*-arsonate; SRBC, sheep erythrocytes; TNP, 2,4,6-trinitrophenyl(ated).

## Materials and Methods

**Preparation of Antigens.** The antigens used in this study are listed in Table I. The synthesis of RAT, DNP-SAC-RAT, (or BI-1), and *N*-2,4-dinitrophenyl-6-amino-caproyl-(proline)<sub>9</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate (DNP-SAC-PRO<sub>9</sub>-RAT; or BI-2) have been previously reported (5, 6).

The antigens BI-2 and *N*-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-*p*-azobenzene-*p*-arsonate-(proline)<sub>10</sub> (DNP-SAC-RAT-PRO<sub>10</sub>; or BI-3) were prepared similarly to the trifunctional antigen, *N*-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-*p*-azobenzene-*p*-arsonate-(proline)<sub>9</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate (DNP-SAC-RAT-PRO<sub>9</sub>-RAT; or TRI). TRI was prepared by the solid-phase synthesis method (7, 8) starting with *N*-tert-butoxyl-carbonyl-tyrosine (BOC-TYR) (OBzCl<sub>2</sub>)-resin and using *N*-tert-butoxyl-carbonyl-(proline)<sub>3</sub>-OH (BOC-PRO<sub>3</sub>-OH) for symmetrical anhydride fragment coupling (9). A detailed description of the synthesis and purification of TRI will be published elsewhere (Manuscript in preparation.).

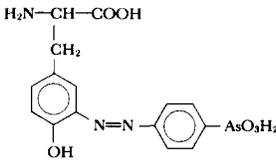
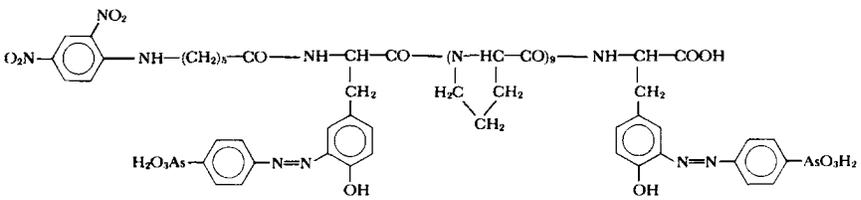
After purification, TRI was characterized by spectrophotometric analysis, amino acid analysis, and elementary analysis for arsenic. Spectra over the range of 300–540 nm showed the expected qualitative and quantitative spectra of DNP and mono-*p*-azobenzene arsenylate chromophores. Amino acid analysis yielded a ratio of proline:tyrosine of 5:1; the expected ratio is 4.5:1. The elementary analysis was found to be 6.92% for arsenic; the calculated value was 7.4%.

*Animals and Immunization*

**MICE.** Female A/J (H-2<sup>a</sup>) mice 6–8 wk of age were purchased from The Jackson Laboratory, Bar Harbor, Maine. Mice used in these experiments were from 2 to 8 mo old.

**IMMUNIZATION.** The antigens were dissolved in phosphate-buffered saline (PBS) at 2 mg/ml and emulsified with an equal volume of complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, Mich.). Typically, each A/J mouse received a single injection of 100 μg of antigen intraperitoneally in 0.1 ml for either priming or boosting. Mice were boosted with the

TABLE I  
The antigens

|  |  |             |                             |             |  |
|--|--|-------------|-----------------------------|-------------|--|
| MONOFUNCTIONAL:  | RAT, L-tyrosine- <i>p</i> -azobenzene- <i>p</i> -arsonate.   |             |                             |             |  |
|  |   |             |                             |             |  |
| BIFUNCTIONALS:   | BI-1, DNP-SAC-RAT, <i>N</i> -2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine- <i>p</i> -azobenzene- <i>p</i> -arsonate.<br>BI-2, DNP-SAC-PRO <sub>9</sub> -RAT, <i>N</i> -2,4-dinitrophenyl-6-amino-caproyl-(proline) <sub>9</sub> -L-tyrosine- <i>p</i> -azobenzene- <i>p</i> -arsonate.<br>BI-3, DNP-SAC-RAT-PRO <sub>10</sub> , <i>N</i> -2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine- <i>p</i> -azobenzene- <i>p</i> -arsonate-(proline) <sub>10</sub> . |             |                             |             |  |
| TRIFUNCTIONAL:   | TRI, DNP-SAC-RAT-PRO <sub>9</sub> -RAT, <i>N</i> -2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine- <i>p</i> -azobenzene- <i>p</i> -arsonate-(proline) <sub>9</sub> -L-tyrosine- <i>p</i> -azobenzene- <i>p</i> -arsonate.   |             |                             |             |  |
| HAPTEN DNP   | SPACER SAC   | CARRIER RAT | SPACER PROLINE <sub>9</sub> | CARRIER RAT |  |
|  | (8 Å)  |             | (28 Å)                      |             |  |
|  |  |             |                             |             |  |

All amino acids are of L-configuration.

appropriate antigen 30 d after priming. Control mice were injected with 0.1 ml of emulsion prepared from equal volumes of PBS and CFA.

*PFC Assay.* Goat anti-mouse IgM (heavy chain specific) and rabbit anti-mouse IgG (heavy and light chain specific) antisera were purchased from N. L. Cappel Laboratories, Inc., Cochranville, Pa. Guinea pig complement, fetal calf serum (FCS), Hanks' balanced salt solution (HBSS), and minimum essential medium (MEM) were purchased from Grand Island Biological Co., Grand Island, N. Y.

Anti-DNP PFC were assessed using 2,4,6-trinitrophenylated (TNP)-sheep erythrocytes (SRBC) as indicator cells (10) in a modification of the Jerne plaque assay (11). It has been shown that equal numbers of anti-DNP PFC were detected in the same experiment using either DNP-SRBC or TNP-SRBC as indicator cells (12). Because of the ease of preparation, TNP-SRBC were used in all the experiments and the results were expressed as the number of anti-TNP PFC.

On the day of PFC assay, single-spleen-cell suspensions were prepared with HBSS at a final concentration of 1 spleen/2 ml. 0.1 ml of TNP-SRBC (diluted 1:6) and 0.1 ml of the appropriate spleen cell suspension were added to 2 ml of 0.7% agar in MEM and the mixture was plated in 9-cm plastic Petri dishes. After incubating the dishes at 37°C for 1 h, 2 ml of complement (guinea pig serum diluted appropriately in PBS) was added to each dish. The dishes were incubated at 37°C for another hour, the complement was poured off, and the PFC were counted.

IgG PFC were detected by first inhibiting IgM PFC with anti-IgM anti-serum, which was added to the cells before the plates were poured. Then, the IgG PFC were developed by adding anti-IgG antiserum with complement to the dishes after a 1-h incubation (13).

*Statistics.* Because the PFC responses to these antigens were relatively weak, careful statistical analysis of the data was necessary. Groups of five mice were used in each experiment except when indicated otherwise. The PFC responses of individual mice were analyzed and recorded separately. It has been demonstrated that the logarithm of PFC responses of individual mice follows a normal distribution (14). Therefore, the geometric means (GM; which are the arithmetic means of the log of the individual responses of the groups) were calculated and expressed in both Figures and Tables. SE (SD of the means of samples of  $n$  variates) were calculated using the equation  $SE = (n)^{1/2} \cdot (n/n-1)^{1/2} \cdot s$ ; where  $s$  is the SD of the sample (15). The upper limit of the GM is  $\log^{-1} (\log GM + SE) = \log^{-1} (\log GM) \times \log^{-1} (SE) = GM \times \log^{-1} SE$ . By the same method, the lower limit of the GM is  $GM \div \log^{-1} SE$ . Therefore, the form  $GM \times / \div \log^{-1} SE$  is used in all Figures and Tables to express the GM and its upper and lower limits.

The significance of experimental results were determined by the parametric Student's  $t$  test. In addition, the nonparametric Mann-Whitney U test (i.e., Wilcoxon's test for the unpaired cases) was also performed for further confirmation. A two-tailed table was used with  $P < 0.05$  considered significant and  $P < 0.01$  considered highly significant.

## Results

*Primary Antibody Responses to BI-1 (DNP-SAC-RAT) and TRI.* Immunization of A/J mice with either BI-1 or TRI (100  $\mu$ g in CFA) led to significant direct anti-TNP PFC responses; 4,074 PFC/spleen by BI-1 vs. 2,115 PFC/spleen by CFA control on day 13, and 12,477 PFC/spleen by TRI vs. 6,262 PFC/spleen by CFA control on day 7 (Figs. 1 and 2). The 100- $\mu$ g dose used for both antigens was based on a preliminary dose-kinetics study which demonstrated that this dose was optimal among the various doses tested (e.g., 1, 10, 100, and 1,000  $\mu$ g) when PFC responses were assayed on day 7 and day 10 after priming. The high response by the CFA control group in Fig. 1 was an exception to the average responses of CFA controls in all experiments (2,000–3,000 PFC/spleen). Both responses were extremely weak and consisted exclusively of IgM PFC. Their detection required the plaquing of large numbers of cells (1/20 spleen equivalent). The significance of both responses was shown by the two-tailed

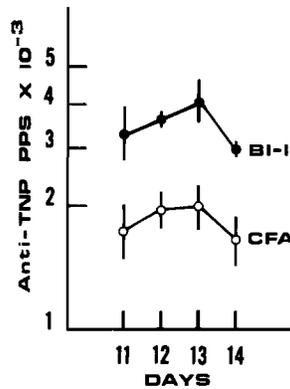


FIG. 1. The primary direct anti-TNP PFC response to BI-1. Groups of five mice each (in this and all subsequent figures) were immunized intraperitoneally with 100  $\mu$ g of BI-1 in CFA, or with PBS in CFA. The GM and their upper and lower limits are expressed in this and all subsequent figures. Solid circles represent BI-1-immunized mice and open circles represent CFA-injected mice. PPS, PFC per spleen. Responses earlier than 11 d were monitored in one experiment, which showed no significant response to BI-1 (e.g., 2,481 PPS by BI-1 vs. 1,894 PPS by CFA control on day 5; 2,795 PPS by BI-1 vs. 2,042 PPS by CFA control on day 7; data not shown.)

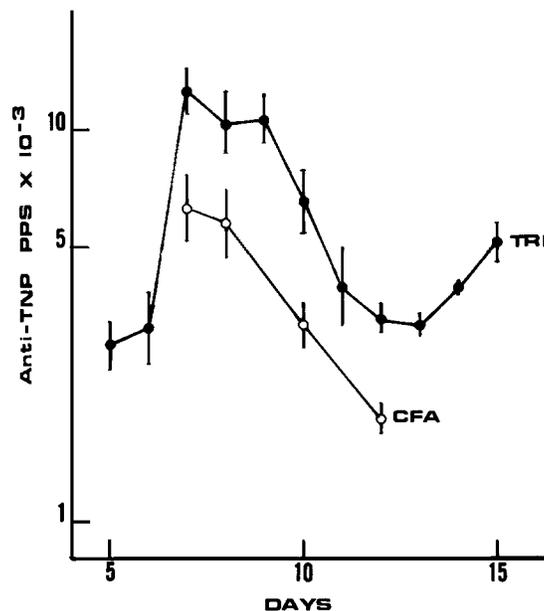


FIG. 2. The primary direct anti-TNP PFC response to TRI. Solid circles represent TRI-immunized mice and open circles represent CFA-injected mice.

Student's *t* test and the two tailed nonparametric Mann-Whitney U test ( $P < 0.01$  at some time points and  $P < 0.05$  at others). Because these antigens are very small molecules (BI-1, 688 mol wt; TRI, 1,954 mol wt), it was considered that the kinetics of the antibody responses might be different from those to classical proteins or haptenated protein antigens. Accordingly, the responses to BI-1 were followed daily from day 5 to day 29, and responses to TRI were followed daily from day 5 to day 27 after immunization in two separate experiments (partial results shown in Figs. 1 and

2). No significant anti-TNP IgG PFC responses were observed throughout the entire period. For anti-TNP IgM PFC, the response to BI-1 reached peak levels between day 9 and 13, and the response to TRI peaked between day 7 and 9. In subsequent experiments, primary responses were followed from day 7 to 14 after immunization.

There was no qualitative difference found between the primary responses to BI-1 and TRI in that only direct PFC were observed. However, there was a small but significant quantitative difference. The peak responses to the two antigens in four separate experiments were as follows (plaques per spleen expressed as the GM of five individual mice): 4,899 (day 11) and 4,911 (day 13) to BI-1; 12,477 (day 7) and 8,596 (day 7, four mice only) to TRI. To confirm these small differences in the kinetics and intensity of the IgM PFC responses to the two antigens, they were compared in a group of mice immunized simultaneously in two experiments. The results confirmed that TRI did, indeed, induce an earlier and about twofold higher response than BI-1 (10,125  $\times$   $\pm$  1.27 PFC/spleen on day 8 to TRI vs. 6,322  $\times$   $\pm$  1.16 PFC/spleen on day 11 to BI-1). Pooling the results of six separate experiments, the GM of PFC per spleen on peak days were 5,016  $\times$   $\pm$  1.10 for BI-1 and 10,002  $\times$   $\pm$  1.10 for TRI. The difference was highly significant ( $P < 0.01$ ) by the two tests employed.

*Secondary Antibody Responses to BI-1 and TRI.* A/J mice were primed and boosted 1 mo later with 100  $\mu$ g of BI-1 or TRI in CFA, and their anti-TNP PFC of both IgM and IgG classes were followed from day 4 to day 9 or 10 after secondary injection. Although only quantitative differences in the primary anti-TNP responses to BI-1 and TRI were found, both quantitative and qualitative differences between the secondary anti-TNP PFC responses to the two antigens were observed (Fig. 3). Although no IgG PFC appeared in the secondary response to BI-1, both IgM and IgG PFC appeared in the secondary response to TRI. The anti-TNP IgG PFC of the

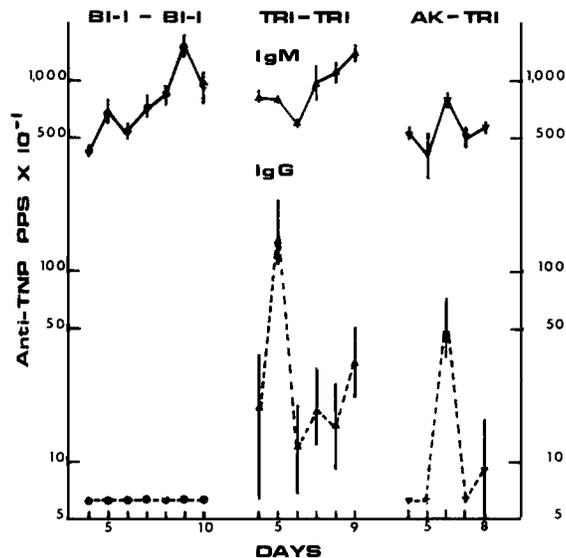


FIG. 3. The secondary anti-TNP PFC responses to BI-1 and TRI. Circles represent BI-1-primed and BI-1-boosted mice; triangles represent TRI-primed and TRI-boosted mice; inverted triangles represent ABA-KLH-primed and TRI-boosted mice; solid lines represent IgM PFC responses; dashed lines represent IgG PFC responses.

secondary response to TRI reached  $1,615 \times / \pm 1.72$  PFC/spleen on day 5 after boosting, compared with the background responses to BI-1 of  $\sim 50$  PFC/spleen followed from day 2 to d 11 after boosting with BI-1.

Consistent and significant anti-azobenzene-*p*-arsonate (ABA) PFC responses have not been observed in A/J mice immunized with BI-1 or TRI, whereas substantial anti-ABA PFC ( $\sim 50,000$  PFC/spleen) have been detected in mice immunized with  $500 \mu\text{g}$  ABA-keyhole limpet hemocyanin (KLH) in CFA (data not shown). Because the existence of antibody-dependent helper T cells has been suggested (8), ABA-KLH-primed mice were included in the initial experiment to increase the probability of eliciting putative antibody-dependent help, and, hence, to assess its possible role in other responses. The results (Fig. 3) showed that both anti-TNP IgM and IgG PFC were detected in mice boosted with TRI, regardless of whether they were primed with TRI or ABA-KLH. The anti-TNP IgM response peaked at a level of  $\sim 10,000$  PFC/spleen, which was about the same as the primary anti-TRI response (Fig. 2). The anti-TNP IgG PFC reached  $1,615$  PFC/spleen on day 5 in TRI-primed mice, and  $500$  PFC per spleen on day 6 in ABA-KLH-primed mice. However, there was no anti-TNP IgG PFC response in mice boosted with BI-1 after priming with BI-1 or ABA-KLH (data not shown), in spite of there being about the same number of IgM PFC as in mice boosted with TRI. The IgG PFC responses were highly significant ( $P < 0.01$  by both parametric and nonparametric tests) in TRI-primed mice on days 5, 7, and 9, and in ABA-KLH-primed mice on day 6; and were significant ( $P < 0.05$  by both tests) in TRI-primed mice on days 6 and 8 (Fig. 3). The peak IgG PFC responses were  $\sim 80$  times background in TRI primed mice and 25 times background in ABA-KLH-primed mice.

It should be pointed out that mice primed and boosted with BI-1 made a higher secondary than primary anti-TNP IgM PFC response. This suggested a degree of immunological memory differing from that of mice primed and boosted with TRI, in which the secondary anti-TNP IgM PFC response was not higher, but an IgG PFC response emerged.

To further investigate the critical requirements for inducing IgG PFC responses, mice were primed with TRI and boosted with BI-1 or were primed with BI-1 and boosted with TRI. Assays from days 3 to 12 revealed that anti-TNP IgG PFC only appeared in mice boosted with TRI, not in those primed with TRI and boosted with BI-1 (partial results shown in Fig. 4). The IgG PFC responses in TRI-boosted mice were highly significant ( $P < 0.01$  by both tests) from day 6–11 after boosting, and the peak response ( $427$  PFC/spleen) was  $\sim 20$ -fold higher than background responses in BI-1-boosted mice.

The induction of anti-TNP IgG PFC in mice primed with BI-1 and boosted with TRI (Fig. 4) suggested that it is unlikely that BI-1 induced Ig class-specific and antigen-specific suppressor T cells that were responsible for the absence of anti-TNP IgG PFC responses after secondary challenge with BI-1. Moreover, the induction of IgG PFC in mice primed with ABA-KLH and boosted with TRI (Fig. 3) indicated that DNP-primed B cells were not necessary for the induction of anti-TNP IgG PFC responses.

*Minimum Requirements for Induction of IgG Antibody Responses by TRI.* To further determine the minimum requirements for inducing IgG PFC in this relatively simple and well-characterized system, mice were primed with RAT (409 mol wt), a mono-

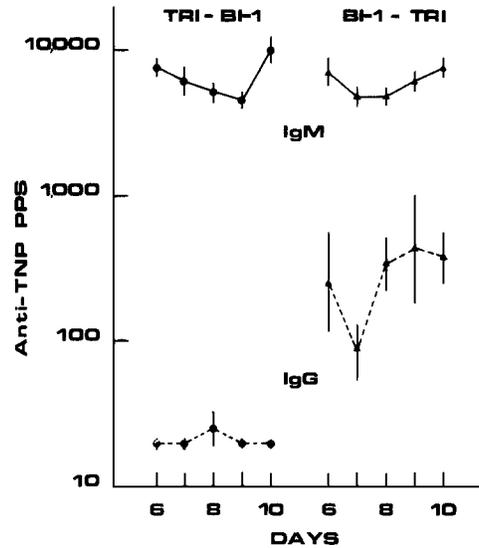


FIG. 4. Anti-TNP PFC responses of mice primed and boosted with different antigens as indicated. Circles represent TRI-primed and BI-1-boosted mice; triangles represent BI-1-primed and TRI-boosted mice; solid lines represent IgM PFC responses; dashed lines represent IgG PFC responses.

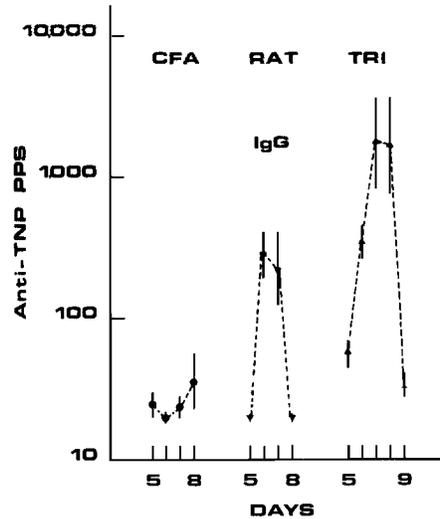


FIG. 5. Anti-TNP PFC responses of mice primed with different antigens and boosted with TRI. Only IgG PFC responses are expressed. Circles represent CFA-injected mice; inverted triangles represent RAT-primed mice; triangles represent TRI-primed mice.

functional T cell immunogen. These mice were then boosted with TRI and their responses were followed from day 5 to 10 in two separate experiments (partial results of one experiment shown in Fig. 5). Significant anti-TNP IgG PFC responses were given by RAT-primed mice on days 6 and 7 ( $P < 0.01$ ), whereas no anti-TNP IgG PFC responses were detected in CFA-injected and TRI-boosted mice, which mounted about the same anti-TNP IgM PFC responses as those of RAT-primed mice. However, the IgG PFC responses were much weaker than those of TRI-primed mice (used as

the positive control),  $278 \times / \pm 1.47$  PFC/spleen by RAT-primed mice vs.  $1,719 \times / \pm 2.1$  PFC/spleen by TRI-primed mice (Fig. 5). The differences were highly significant on day 8 ( $P < 0.01$ ) and significant on day 10 ( $P < 0.05$  by the Student's  $t$  test only). In addition, no significant anti-TNP IgG PFC were detected in mice primed with RAT, boosted with BI-1, and followed from day 5 to 7 (one experiment) or day 5 to 9 (one experiment) (data not shown).

These results (Figs. 3–5) indicate that the minimum requirements for IgG antibody induction are carrier priming with a mono-epitope carrier (e.g., RAT), and boosting with an antigen that has a minimum of two carrier determinants. Moreover, the weaker anti-TNP IgG PFC responses in RAT-primed mice than in mice primed with BI-1 or TRI suggested that DNP-priming can significantly enhance the anti-TNP IgG PFC response, although it was not a necessity.

*Role of the Spacer in IgG Induction.* Although BI-1 (DNP-SAC-RAT) induced only IgM anti-TNP PFC even after boosting, TRI induced both IgM and IgG PFC after priming and boosting (Fig. 3). Moreover, anti-TNP IgG PFC appeared in mice primed with RAT or BI-1 and boosted with TRI (Figs. 4 and 5). Thus, it was of interest to determine the critical features in the structures of BI-1 and TRI responsible for the induction of IgG PFC responses.

The major structural differences between TRI and BI-1 include: (a) two RAT carrier epitopes in TRI vs. one RAT carrier epitope in BI-1; (b) a sequence of nine proline residues as spacer in TRI vs. no proline residues in BI-1; and (c) a distance of  $\sim 36 \text{ \AA}$  between the DNP haptenic epitope and one carrier epitope in TRI (SAC-RAT-PRO<sub>9</sub>) vs. the maximum span of  $8 \text{ \AA}$  of the SAC spacer in BI-1. To define the critical factor(s) that activate(s) IgG PFC, additional model antigens with selected properties were prepared, as follows: (a) DNP-SAC-PRO<sub>9</sub>-RAT (BI-2), with a spacer consisting of nine proline residues plus a SAC group, thereby simulating the spacer between DNP and RAT in TRI; (b) DNP-SAC-RAT-PRO<sub>10</sub> (BI-3), in which a proline residue was substituted for the second RAT moiety of TRI. These two compounds provide bifunctional antigens with spacers that are found in TRI. They were used to assess whether the IgG response induced by TRI was a result of the second RAT epitope or to the arrangement of the spacer groups.

The earlier results showed that priming with TRI induced the strongest IgG PFC responses, and that the responses appeared between day 5 and 9 after boosting (Figs. 3–5). Therefore, mice were primed with TRI, boosted with appropriate antigens, and followed from day 5 to 9 to detect anti-TNP IgG PFC responses induced by BI-2 and BI-3 (partial results shown in Fig. 6). It can be seen that BI-2 induced anti-TNP IgG PFC responses in TRI-primed mice, whereas BI-3 did not. The anti-TNP IgG PFC response by BI-2-boosted mice reached  $2,462 \times / \pm 2.20$  PFC/spleen on day 5 and  $1,915 \times / \pm 1.33$  PFC/spleen on day 8, in contrast to the background response by BI-3-boosted mice of only  $\sim 70$  PFC/spleen throughout the period. However, BI-3 induced significant anti-TNP IgM PFC:  $6,778 \times / \pm 1.10$  PFC/spleen vs.  $2,910 \times / \pm 1.22$  PFC/spleen in CFA-injected mice (data not shown;  $P < 0.01$  by both tests). Moreover, BI-2 and BI-3 induced about the same anti-TNP IgM PFC responses:  $6,778 \times / \pm 1.10$  PFC/spleen by BI-3 vs.  $3,917 \times / \pm 1.06$  PFC/spleen by BI-2 in one experiment (Fig. 6); and  $4,244 \times / \pm 1.09$  PFC/spleen by BI-3 vs.  $4,948 \times / \pm 1.09$  PFC/spleen by BI-2 in another experiment (data not shown).

Because mice primed with TRI gave about equivalent anti-TNP IgG PFC responses

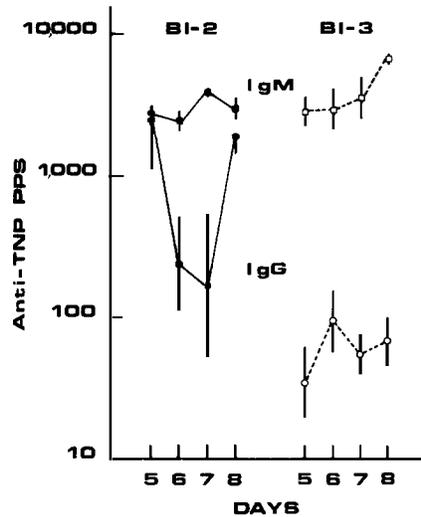


FIG. 6. Anti-TNP PFC responses of mice primed with TRI and boosted with the indicated antigens. Solid lines represent BI-2 boosted mice; dashed lines represent BI-3 boosted mice; squares represent IgG PFC responses; circles represent IgM PFC responses.

TABLE II  
Secondary Anti-TNP IgG PFC Responses to TRI and BI-2 in Mice Primed with RAT or Ac-PRO<sub>10</sub>-RAT\*

| Days‡ | RAT-primed and TRI-boosted |              | RAT-primed and BI-2-boosted |              | Ac-PRO <sub>10</sub> -RAT-primed and BI-2-boosted |              |
|-------|----------------------------|--------------|-----------------------------|--------------|---|--------------|
|       | Experiment 1               | Experiment 2 | Experiment 3                | Experiment 4 | Experiment 3                                      | Experiment 5 |
| 5     | 10                         | 252          | 14                          | 14           | 27  | 55           |
| 6     | 252                        | 80           | 37                          | 20           | 74  | 25           |
| 7     | 225                        | 61           | 42                          | 19           | 225   | 35           |
| 8     | 10                         | 28           | ND§                         | 34           | 109   | 216          |

\* Groups of five mice each were primed with 100 µg of RAT or Ac-PRO<sub>10</sub>-RAT in CFA and boosted with 100 µg of TRI or BI-2 in CFA 1 mo later. The GM of IgG PFC/spleen are expressed.

‡ Days after boosting.

§ ND, not done.

when boosted with BI-2 or TRI (Figs. 3 and 6; and additional data not shown), it was of interest to determine if priming with TRI was essential for the IgG response to BI-2. As observed earlier, priming with RAT and boosting with TRI gave rise to anti-TNP IgG PFC (Fig. 5). Accordingly, mice were primed with RAT and boosted with BI-2 and BI-3. In contrast to boosting with TRI, neither of the bifunctional antigens induced anti-TNP IgG PFC responses in mice primed with RAT. The data for BI-2 are shown in Table II. Thus, BI-2 and TRI are not equivalent in their capacity to induce IgG antibody responses.

Our interpretation of these findings is that priming with TRI could induce anti-polyproline antibody, which might cross-link BI-2 molecules into aggregates consisting of at least two BI-2 molecules. These poly-BI-2 molecules could then meet the requirement of having at least two carrier epitopes per molecule for inducing IgG antibody responses. Alternatively, polyproline or polyproline-anti-polyproline com-

plexes might function as a second carrier in addition to the RAT carrier, which implies that different carrier-specific helper T cells which recognize different carriers might synergize in activating the IgG antibody response.

To assess the possible formation of anti-polyproline antibodies and their role(s) in inducing anti-TNP IgG PFC, another antigen, acetyl-(proline)<sub>10</sub>-L-tyrosine-*p*-aminobenzene-*p*-arsonate (Ac-PRO<sub>10</sub>-RAT), was prepared (16). Mice were primed with Ac-PRO<sub>10</sub>-RAT and boosted with BI-2. It can be seen (Table II) that significant anti-TNP IgG PFC responses were detected ( $P < 0.05$  compared with mice primed with RAT or CFA, and boosted with BI-2; by the two-tailed Student's *t* test only). Moreover, this IgG response was equivalent to the anti-TNP IgG PFC response induced by RAT-priming and TRI-boosting (Table II).

The possible induction of anti-polyproline antibodies and their effect(s) were further assessed by serum transfer experiments. Mice were primed with Ac-PRO<sub>10</sub>-RAT in CFA and their putative anti-polyproline anti-sera were collected 1 mo later. Mice that had been primed 1 mo earlier with RAT in CFA were injected intraperitoneally with various quantities of the putative antipolyproline antisera and immunized simultaneously with BI-2 in CFA. The results from one experiment, followed from day 5 to 8 after challenge, were equivocal. As described earlier, the anti-TNP IgG PFC responses induced by either RAT-priming with TRI-boosting or Ac-PRO<sub>10</sub>-RAT-priming with BI-2-boosting (Table II) were extremely weak and on the borderline of significance. This suggested that a large number of mice had to be used, a minimum of 10/day (including 5 mice as controls) for a minimum period of 4 d, to detect responses of this magnitude. The cost of this already risky series of experiments was further increased by the large number of mice used to prepare and titrate the putative antipolyproline antisera. Together, these factors made it unrealistic to further pursue the possible role of anti-polyproline antibodies in the IgG responses by serum transfer experiments.

To test the possible carrier function of polyproline in BI-2, another new antigen, *N*-2,4-dinitrophenyl-6-amino-caproyl-(proline)<sub>9</sub>-L-tyrosine (DNP-SAC-PRO<sub>9</sub>-TYR), was used. This compound is an intermediate in the synthesis of BI-2, used for the last step of diazonium coupling. DNP-SAC-PRO<sub>9</sub>-TYR did not induce significant anti-TNP antibody responses of either class, either in primary responses (followed from day 6 to 11 after priming) or secondary responses (followed from day 5 to 9 after boosting) in mice primed with TRI in CFA 1 mo earlier (data not shown). These results suggest that the polyproline spacer in BI-2 does not function as a carrier. The cumulative findings favor, but do not prove, the interpretation that IgG induction by BI-2 is mediated by anti-prolyl antibody. The failure of such antibody to mediate IgG induction by BI-3 is unclear, but may be related to spatial features of the molecule that prevent aggregation.

### Discussion

The central conclusion to be drawn from these experiments is that the minimum requirement for IgG antibody formation in mice is priming with a monofunctional carrier and boosting with a trifunctional antigen consisting of two carrier epitopes and a single haptenic epitope. The anti-hapten IgG PFC responses resulting from this regimen were extremely weak, requiring the plaquing of large numbers of spleen cells for their unequivocal detection. Parenthetically, it should be noted that priming with

more complex antigens bearing ABA epitopes (BI-1 and ABA-KLH) sufficed as well or better for IgG responses, the essential factor being boosting with a molecule bearing at least two carrier epitopes. This was exemplified by the inability of BI-1 to induce IgG responses in mice primed with TRI, whereas the converse engendered IgG PFC. Primary responses to bi- and trifunctional antigens were exclusively IgM; IgG PFC were observed only in secondary responses. We interpret this as a requirement for the expansion of helper T cells, although ABA-specific B cells are also expanded by RAT (2) and may play a role in the development of IgG anti-hapten responses, as discussed below. It is apparent that expansion of hapten-specific B cells is not required for IgG responses, because RAT sufficed as a priming agent for anti-DNP IgG PFC.

An exception to the general requirement for two carrier epitopes for IgG responses was apparently made by the IgG response in mice boosted with the bifunctional compound BI-2. This compound, as well as BI-3, was synthesized to assess the role, if any, of the polyproline spacer in TRI in isotype switching. The combined evidence from a number of experiments supports the interpretation that anti-prolyl antibodies were responsible for the effect, perhaps by cross-linking BI-2 into aggregates which would then, in effect, possess the plurality of carrier epitopes required for IgG production. For one thing, anti-prolyl antibodies were induced in guinea pigs immunized with the bifunctional antigen *N*-2,4-dinitrophenyl-(proline)<sub>10</sub>-L-tyrosine-*p*-aminobenzene-*p*-arsonate (DNP-PRO<sub>10</sub>-RAT), demonstrating that the decaproline spacer also served as a haptenic determinant (17). The most telling observations in the present series of experiments involved the inability of BI-2 to induce IgG responses unless proline-containing antigens were used for priming. Thus, BI-2 induced only IgM PFC in mice primed with RAT, but induced IgG as well as IgM PFC in animals primed with Ac-PRO<sub>10</sub>-RAT (Table II). Attempts were made to firmly establish the antibody mechanism by transferring serum from mice immunized with Ac-PRO<sub>10</sub>-RAT into RAT-primed recipients, which were then challenged with BI-2. The results of the transfer experiments, as might be anticipated in view of the marginal response to these antigens, were inconclusive. Hence, the findings strongly suggested, but failed to conclusively prove, the antibody mechanism for isotype switching by BI-2. In any event, the ability of TRI coupled with the inability of BI-2 to induce IgG PFC in mice primed with RAT is viewed as strong support for the conclusion that at least two carrier epitopes are indeed required for isotope switching.

A remaining paradox was the failure of BI-3 to induce IgG PFC in mice primed with TRI (Fig. 6). The only apparent difference between BI-2 and BI-3 is the location of the polyproline spacer (Table I). If cross-linking by anti-prolyl antibody was indeed responsible for the IgG response to BI-2, it is difficult to visualize why BI-3 should be different in this respect. One consideration that comes to mind is the critical displacement between haptenic and carrier epitopes for antibody responses (17). Because BI-2 and BI-3 induced about equal IgM PFC responses, the difference in IgG induction implies that spatial requirements for IgM and IgG responses may differ. However, the experimental evidence is insufficient to resolve this question.

Upon considering plausible mechanisms by which dual carrier epitopes dictate isotype switching by B cells, the obvious alternatives are: (a) more efficient focusing of a single type of T cell help versus (b) the mediation of different synergizing forms of help. The recent literature suggests that a single type of help, whether antigen-specific or idiotypic-specific, triggers only IgM antibody responses. For one thing,

enriched antigen-specific helper T ( $T_H$ ) cells from long-term cultures induced B cells from nude mice to secrete IgM, but not IgG, antibodies in vivo (18) and in vitro (19). For another, injection of serum antibodies induced direct PFC of the same specificity in normal mice, but not in nude mice, suggesting that  $T_H$  cells were involved (20). Moreover, monoclonal antibodies likewise induced direct PFC of identical specificity, whereas variant hybridoma products, in which the light chain came from the myeloma parent, were inactive, indicating that the  $T_H$  cells involved were specific for self-Ig determinants. These findings, together with those supporting the existence of synergistic help (4), provide a compelling argument for the combined action of different forms of help in isotype switching.

Accordingly, a model can be formulated in which, on a molecule of TRI bound by a DNP-specific B cell, one of the RAT epitopes focuses ABA-specific help, presumably in the form of a soluble mediator, whereas the other RAT epitope binds anti-ABA antibody, which in turn serves a determinant to focus the Ig-specific help. In this regard, the requirement for RAT-priming in the IgG response may be for the purpose of an early secondary anti-ABA antibody response rather than, or in addition to, the priming of helper T cells, because expansion of ABA-specific B cells in RAT-immunized mice has been demonstrated (2).

Because a B cell has many Ig receptors on its surface, it is likely that it may have many antigen molecules on its surface. In that case, a molecule with a single carrier epitope, such as BI-1, should be able to focus both helper factors on the surface of an individual cell. Why, then, doesn't BI-1 induce IgG responses? One possibility is that a given B cell may capture very few antigen molecules, particularly because these small molecules are likely to be cleared rapidly. Alternatively, the synergistic signals may not operate through distant receptors on a B cell surface, but may require delivery in the immediate vicinity of the same hapten-binding receptor. At this point, one can only speculate about the detailed mechanisms of B cell activation and immunoglobulin isotype switching.

### Summary

L-Tyrosine-*p*-azobenzene-*p*-arsonate (RAT) is immunogenic and serves as a carrier for anti-hapten antibody responses in guinea pigs, rats, and mice. However, the murine anti-*N*-2,4-dinitrophenyl (DNP) plaque-forming cell (PFC) response to the bifunctional antigen 2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-*p*-azobenzene-*p*-arsonate (DNP-SAC-RAT; or BI-1) is extremely weak (2,000–4,000 PFC/spleen) and exclusively IgM in both primary and secondary responses. The 6-amino-caproyl group serves as a spacer in this antigen between the DNP haptenic and RAT carrier epitopes.

In view of recent evidence indicating that different T helper cells synergize for optimal antibody responses, a trifunctional antigen, *N*-2,4-dinitrophenyl-6-amino-caproyl-L-tyrosine-*p*-azobenzene-*p*-arsonate-(proline)<sub>9</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate (DNP-SAC-RAT-PRO<sub>9</sub>-RAT; or TRI), was prepared to investigate the effect of adding a second RAT epitope to BI-1. The nonaproline spacer between the two RAT epitopes in TRI is assumed to be a rigid rod of ~28 Å. TRI induced about twice as many PFC as BI-1 in primary responses of A/J mice, and induced both IgM and IgG PFC in secondary responses. Furthermore, TRI induced IgG PFC responses in mice primed with *p*-azobenzene-*p*-arsonate-keyhole limpet hemocyanin, BI-1, or RAT, whereas boosting with BI-1 failed to induce IgG PFC, even in mice primed with TRI.

These findings indicate that the minimum antigen structural requirements for inducing IgG PFC in mice are two carrier epitopes and one haptenic epitope. In addition, priming with a mono-epitope carrier (RAT) is sufficient preparation for IgG responses to a trifunctional immunogen.

Because TRI differs from BI-1 by the (proline)<sub>9</sub> spacer as well as the additional RAT epitope, two other compounds, *N*-2,4-dinitrophenyl-6-amino-caproyl-(proline)<sub>9</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate (DNP-SAC-PRO<sub>9</sub>-RAT; or BI-2) and *N*-2,4-dinitrophenyl-6-amino-caproyl-(proline)<sub>9</sub>-L-tyrosine-*p*-azobenzene-arsonate (DNP-SAC-RAT-PRO<sub>10</sub>; or BI-3), were prepared to evaluate the possible role of the spacer in the observed responses. BI-2, but not BI-3, induced IgG as well as IgM PFC in TRI-primed mice. However, BI-2 failed to induce IgG responses in RAT-primed mice, indicating that TRI and BI-2 were not equivalent immunogens. Because anti-prolyl antibodies had been found in guinea pigs immunized with *N*-2,4-dinitrophenyl-(proline)<sub>10</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate (DNP-PRO<sub>10</sub>-RAT), it seemed possible that priming with TRI might induce anti-prolyl antibodies, which, in turn, could cross-link BI-2 molecules into aggregates containing at least two carrier epitopes. To help resolve this question, mice were immunized with acetyl-(proline)<sub>10</sub>-L-tyrosine-*p*-azobenzene-*p*-arsonate and boosted with BI-2. IgG PFC responses were detected, suggesting that anti-prolyl antibodies were indeed responsible, because priming with RAT and boosting with BI-2 did not induce IgG formation.

Accordingly, the observations that IgG responses in RAT-primed mice were induced only by TRI and not by any of the bifunctional antigens indicate that two carrier epitopes per antigen molecule are indeed required for IgG induction. They also provide indirect evidence for synergistic help in the switching of immunoglobulin isotypes.

We thank Miss Inge M. Stoltenberg for excellent technical assistance.

*Received for publication 7 July 1980 and in revised form 13 August 1980.*

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