THE ALLOGENEIC EFFECT IN INBRED MICE

III. UNIQUE ANTIGENIC STRUCTURAL REQUIREMENTS IN THE EXPRESSION OF THE PHENOMENON ON UNPRIMED CELL POPULATIONS IN VIVO

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The allogeneic effect, as initially described in guinea pigs (1-3) and more recently extended to inbred mice (4-6), demonstrates that the transfer of immunocompetent lymphoid cells to animals previously primed with a hapten-carrier conjugate, such as 2,4-dinitrophenyl (DNP)1-keyhole limpet hemocyanin (KLH), markedly enhances the antihapten antibody response to an appropriately timed secondary challenge with that hapten coupled to an unrelated carrier (for review, see reference 7). This phenomenon requires the initiation of an active graft-vs.-host (GVH) reaction in the lymphoid tissue of the primed host, and reflects the direct interaction of the allogeneic donor thymus-derived (T) cells with primed host bone marrow-derived (B) lymphocytes, irrespective of the presence of host T cells (5). Further, for the wide range of antigens heretofore employed, it has been shown to be essential that the host cell population be primed before the administration of allogeneic cells (1-4, 7). In our hands, numerous attempts with a variety of immunogenic molecules (or conjugates) have failed to show enhanced primary responses as a result of transfer of allogeneic cells (1-3), and these conditions frequently caused suppression of the primary response (4, 7). In contrast, Ordal and Grumet have recently described experiments in which the induction of a GVH reaction enabled genetic nonresponder mice to develop primary antibody responses, of the IgG class, to the synthetic polymer poly-t-(Tyr, Glu)-poly-d, l-Ala-poly-l-Lys [(T, G)-A--L] (8). Their observations very clearly indicate the capacity of the allogeneic effect, under certain special circumstances, to influence antibody re-

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1 Abbreviations used in this paper: B, bone marrow-derived; CAF1, (BALB/c X A/J)F1 hybrid mice; DNP, 2,4-dinitrophenyl; DNP-d-GL, DNP conjugate of a synthetic random copolymer of d-glutamic acid and d-lysine; DNP-l-GL, DNP conjugate of a synthetic random copolymer of L-glutamic acid and L-lysine; GVH, graft-vs.-host; KLH, keyhole limpet hemocyanin; MEM, minimal essential medium; NMS, normal mouse serum; OVA, ovalbumin; PFC, plaque-forming cells; SIII, type III pneumococcal polysaccharide; SRBC, sheep erythrocytes; T, thymus-derived; (T, G)-A--L, poly-t-(Tyr, Glu)-poly-d, l-Ala-poly-l-Lys; TNP, 2,4,6-trinitrobenzenesulfonic acid.
sponses in previously unprimed animals, and considered in the context of our repeated failures to observe such effects on primary antibody responses raise important questions concerning the nature of the conditions which permit the expression of the phenomenon in unprimed cell populations.

The present studies were undertaken to investigate the influence of the allogeneic effect on the primary response to the nonimmunogen, DNP-n-GL, a DNP conjugate of a synthetic random copolymer of D-glutamic acid and D-lysine. n-GL is a carrier molecule for which no specific helper T cells exist (2, 9), and conjugated as a DNP copolymer it normally induces a profound state of hapten-specific tolerance in guinea pigs (2) and inbred mice (10). In the experiments described here, we have found that: (a) the allogeneic effect not only prevents the induction of tolerance by DNP-n-GL, but also promotes a striking primary anti-DNP antibody response, which is primarily of the IgG class; (b) the allogeneic effect cannot be demonstrated to enhance primary responses to immunogens such as DNP-ovalbumin (DNP-OVA) or DNP-KLH; and (c) moreover, the removal of carrier-specific T cells for KLH, presumably creating a situation analogous to that for n-GL, does not permit allogeneic lymphoid cells to enhance the primary response to DNP-KLH.

Materials and Methods

Proteins and Hapten-Carrier Conjugates.—Hen OVA, recrystallized five times, was obtained from Pentex Biochemical, Kankakee, Illinois. KLH was purchased from Pacific Bio-Marine Supply Co., Venice, Calif. n-GL was obtained from Pilot Chemicals, Inc., Watertown, Mass. The isomer had an average molecular weight of 30,000 and a ratio of G to L of 60:40. The following DNP conjugates were prepared as previously described (11, 12): DNP9-OVA and DNP14-KLH. The preparation of DNPn-GL has been described elsewhere (2). Subscripts refer to the average number of moles of DNP per mole of carrier for OVA and n-GL and to the number of moles of DNP per 100,000 molecular weight units for KLH.

Animals.—Mice of the inbred line A/J and (BALB/c X A/J)F1 hybrids (CAF1) were obtained from the Jackson Laboratory, Bar Harbor, Maine. All mice were used between 8 and 12 wk of age.

Depletion of T Lymphocytes.—The preparation of anti-θ serum, determination of anti-θ serum cytotoxicity, and anti-θ treatment of spleen cells has been previously described (5).

Cell Transfers and Immunizations.—Varying numbers of semiallogeneic A/J lymphoid cells, obtained as single-cell suspensions, prepared and washed in minimal essential medium (Eagle) (MEM), from spleens of normal donor mice were injected intravenously into non-immune CAF1 recipient mice. CAF1 mice which received no cell transfer served as controls. On the same day or at selected intervals after the cell transfer, groups of recipient and control mice were primarily immunized intraperitoneally with one of several hapten-carrier conjugates. The mice were bled 7, 10, and 14 days after primary immunization and anti-DNP antibody levels were determined as described below. In a number of studies, mice were sacrificed on day 10 and their spleens removed for determination of anti-DNP antibody-producing plaque-forming cells (PFC).

Determination of Anti-DNP Antibody-Producing PFC.—Single-cell suspensions of spleens from individual mice were separately prepared and washed in MEM. Cells from the individual spleens were assayed for IgM and IgG anti-DNP antibody-producing PFC using a modification of the hemolytic plaque technique (13-15). Sheep erythrocytes (SRBC) lightly
conjugated with 2,4,6-trinitrobenzenesulfonic acid (TNP) were used as indicators (16). IgG PFC were developed using a rabbit antimouse immunoglobulin-facilitating serum.

Measurement of Anti-DNP Antibodies.—Serum anti-DNP antibody levels were determined by a modified Farr technique (17, 18) using [3H]DNP-6-amino-N-caproic acid (11). Employing standard curves constructed for individual mouse strains in a manner previously described for inbred guinea pigs (11), percentage of binding was converted into amount of anti-DNP antibody in micrograms per milliliter of serum.

Statistical Analysis.—Serum antibody levels and PFC per spleen were logarithmically transformed and means and standard errors calculated. Group comparisons were made employing Student’s t test. In those mice in which no specific antigen binding could be detected in the serum, a value of 0.10 μg/ml was arbitrarily assigned to allow logarithmic transformation of the data.

RESULTS

Effect of Allogeneic Cell Transfer on the Primary Response to DNP-d-GL.—Groups of normal CAF1 mice were injected intravenously with 25 × 10⁶ normal parental A/J spleen cells, and were then injected intraperitoneally with 100 μg of DNP-d-GL on the same day, 2 days later, or 5 days later. A control group of normal CAF1 mice which received no allogeneic cells was also injected with 100 μg of DNP-d-GL.

As shown in Fig. 1, the control mice, which did not receive allogeneic cells, made no detectable levels of anti-DNP antibody when injected with this dose of DNP-d-GL which has previously been shown to be tolerogenic in mice (10). In contrast, all mice receiving allogeneic cells developed weak anti-DNP antibody responses after challenge with 100 μg of DNP-d-GL. The magnitude of the response obtained was dependent upon the time interval between administration of allogeneic cells and challenge. Thus, the group injected with DNP-d-GL on the same day as cell transfer made the highest antibody response, while an interval of 2 or 5 days between the administration of the allogeneic cells and challenge with DNP-d-GL resulted in significant, but lower, levels of anti-DNP antibody production. Peak antibody production occurred on day 10 after challenge in all groups.

Effect of Varying the Dose of Allogeneic Cells on the Primary Response to DNP-d-GL.—Since d-GL is a nonmetabolizable amino acid copolymer, substantial levels of DNP-d-GL may remain in recipient mice after administration and subsequently bind significant quantities of circulating anti-DNP antibody. The following experiments were therefore designed to determine whether any discrepancy may exist between the actual number of anti-DNP antibody-producing cells (PFC) in the spleen and the quantity of their antibody product in the circulation.

Groups of normal CAF1 mice were injected intravenously with varying numbers of normal parental A/J spleen cells ranging from 10 to 100 × 10⁶ cells per recipient. Normal CAF1 mice which received no allogeneic cells served as controls. All groups were injected intraperitoneally with 100 μg of DNP-d-GL shortly after cell transfer. Serum anti-DNP antibody levels and IgM and IgG PFC in their spleens were assayed 10 days later.
Fig. 1. Enhancement of the primary response to DNP-$\alpha$-GL in CAF1 recipients of parental A strain lymphoid cells. Normal CAF1 mice were injected intravenously with $25 \times 10^6$ A strain spleen cells and challenged intraperitoneally with 100 $\mu$g of DNP-$\alpha$-GL either immediately after cell transfer or 2 or 5 days later. Normal CAF1 mice which received no allogeneic cell transfer and were challenged with 100 $\mu$g of DNP-$\alpha$-GL served as controls. 7, 10, and 14 days after challenge the mice were bled and levels of serum anti-DNP antibody were determined.

The results are presented in Table I and Fig. 2. Unimmunized CAF1 mice, not shown, possessed relatively high levels of background anti-DNP PFC. This high background activity was predominantly directed against the haptenic determinant on the SRBC, since PFC against unconjugated SRBC in the same mice were quite low (165 IgM and 30 IgG). No circulating antibody and only background levels of anti-DNP PFC were detected in control mice (group A). Allogeneic cell recipients, on the other hand, manifested augmented primary responses in terms of both PFC levels and quantities of circulating anti-DNP antibody. The magnitude of the effect was clearly related to the number of allogeneic cells used. The maximum IgM and IgG PFC responses on day 10 were obtained with $50 \times 10^6$ parental cells. However, the fact that somewhat higher levels of circulating anti-DNP antibody occurred in recipients of $100 \times 10^6$ cells suggests that the peak PFC responses of these mice may have
**TABLE I**

**Effect of Varying the Dose of Allogeneic Cells on the Primary Response to DNP-α-GL**

| Group | No. of A/J cells transferred | Primary challenge | Anti-DNP antibody response (day 10) |
|-------|-----------------------------|-------------------|------------------------------------|
|       |                             |                   | IgM | IgG |
| A     | None                        |                   | 1,940 | 3,956 | 0.1 |
| B     | $10 \times 10^6$             |                   | 1,611 | 3,853 | 2.6 |
| C     | $25 \times 10^6$             | $100 \mu g$       | 5,637 | 8,402 | 5.1 |
| D     | $50 \times 10^6$             | DNP-α-GL          | 6,609 | 19,091 | 6.3 |
| E     | $100 \times 10^6$            |                   | 4,498 | 15,536 | 10.7 |

* Groups of normal CAF1 mice were injected intravenously with varying numbers, as indicated, of normal parental A/J spleen cells; CAF1 mice which received no allogeneic cells served as controls. All mice were injected intraperitoneally with $100 \mu g$ DNP-α-GL immediately after cell transfer. Determinations of serum antibody and splenic PFC levels were made 10 days after cell transfer and challenge.

† The data are expressed as geometric means of groups of four mice. A comparison of various groups yielded the following $P$ values. For IgM PFC: comparison of group A with group B, 0.80 > $P$ > 0.70; group A with group C, 0.20 > $P$ > 0.10; group A with group D, 0.005 > $P$ > 0.001; group A with group E, 0.025 > $P$ > 0.02. For IgG PFC: group A with group B, 0.95 > $P$ > 0.90; group A with group C, 0.01 > $P$ > 0.005; group A with group D, 0.001 > $P$; group A with group E, 0.005 > $P$ > 0.001. For serum anti-DNP antibody: comparison of group A with groups B, C, D, and E yielded 0.001 > $P$ in all cases.

![Fig. 2](image-url)  
**Fig. 2.** Enhancement of the primary response to DNP-α-GL by the transfer of varying numbers of allogeneic lymphoid cells. Normal CAF1 mice were injected intravenously with varying numbers of parental A strain spleen cells and challenged intraperitoneally immediately thereafter with $100 \mu g$ of DNP-α-GL. Normal CAF1 mice which received no allogeneic cell transfer and were challenged with $100 \mu g$ of DNP-α-GL served as controls. 10 days after cell transfer and challenge, levels of IgM and IgG anti-DNP splenic PFC were determined.
been reached at an earlier time. The same reasoning probably explains the apparent discrepancy in recipients of $10 \times 10^6$ allogeneic cells which manifested no increase in PFC levels while their circulating anti-DNP antibody responses were clearly higher than controls.

**Effect of Varying the Dose of DNP-p-GL.—**

Groups of normal CAF$_1$ mice either were injected intravenously with $25 \times 10^6$ normal parental A/J spleen cells or received no allogeneic cell transfer. Mice from each group were injected intraperitoneally with varying doses (1, 10, 100, or 500 µg) of DNP-p-GL immediately after cell transfer. Serum anti-DNP antibody levels and IgM and IgG PFC were determined 10 days later.

The results are summarized in Table II and illustrated in Fig. 3. Control mice (groups A, C, E, and G) displayed no detectable circulating anti-DNP antibodies at any dose of DNP-p-GL employed. Such mice did develop low levels of IgM and IgG anti-DNP PFC when injected with 1.0 or 10 µg of DNP-p-GL (groups A and C), whereas the number of PFC, particularly of

| TABLE II |
| --- |
| **Effect of Varying the Dose of DNP-p-GL on the Expression of the Allogeneic Effect** |
|   | Protocol* | Anti-DNP antibody response (day 10): |
|   |   |   |   |   |
|   |   | No. of A/J cells transferred | Primary challenge | PFC/spleen | Serum antibody |
|   |   |   |   | IgM | IgG | µg DNP-p-GL | µg/ml |
| A | None | 0.1 | 2,403 | 2,837 | 0.1 |
| B | $25 \times 10^6$ | 5,636 | 8,710 | 0.7 |
| C | None | 10 | 3,471 | 3,734 | 0.1 |
| D | $25 \times 10^6$ | 7,378 | 21,479 | 32.6 |
| E | None | 100 | 544 | 806 | 0.1 |
| F | $25 \times 10^6$ | 5,229 | 44,137 | 33.0 |
| G | None | 500 | 50 | 2,036 | 0.1 |
| H | $25 \times 10^6$ | 4,470 | 37,267 | 8.9 |

* Groups of normal CAF$_1$ mice were injected intravenously with $25 \times 10^6$ A/J spleen cells. CAF$_1$ mice which received no cell transfer served as controls. All mice were injected with varying doses of DNP-p-GL as indicated immediately after cell transfer. Determination of serum antibody and splenic PFC levels were made 10 days after cell transfer and challenge.

† The data are expressed as geometric means of groups of five mice. A comparison of various groups yielded the following $P$ values. For IgM PFC: comparison of group A with group B yielded $0.05 > P > 0.025$; group C with group D, $0.10 > P > 0.05$; group E with group F, $0.20 > P > 0.10$; group G with group H, $0.025 > P > 0.02$. For IgG PFC: comparison of group A with group B yielded $0.005 > P > 0.001$; group C with group D, $0.001 > P$; group E with group F, $0.05 > P > 0.025$; group G with group H, $0.001 > P$. For serum anti-DNP antibody: comparison of group A with group B, C with D, E with F, and G with H yielded $0.001 > P$ in all cases.
Fig. 3. Enhancement of the primary response to varying doses of DNP-\textsuperscript{\textalpha}-GL by the transfer of allogeneic lymphoid cells. Normal CAF\textsubscript{1} mice were injected intravenously with 25 $\times$ 10$^6$ parental A strain lymphoid cells (solid lines) while control CAF\textsubscript{1} mice received no cell transfer (broken lines). Immediately after cell transfer, mice from both groups were challenged intraperitoneally with varying doses of DNP-\textsuperscript{\textalpha}-GL. 10 days after cell transfer and challenge, both serum anti-DNP antibody levels and IgM and IgG anti-DNP splenic PFC were determined.

IgM class, were considerably diminished below background in control mice injected with 100 or 500 $\mu$g of DNP-\textsuperscript{\textalpha}-GL (groups E and G). The higher doses, it should be noted, have been shown to be tolerogenic (10). In contrast, allogeneic cell recipients developed enhanced primary anti-DNP responses which were related in magnitude to the amount of DNP-\textsuperscript{\textalpha}-GL administered up to the 100 $\mu$g dose. The most striking effects were reflected in the anti-DNP PFC of the IgG class and the level of circulating antibodies. Increasing the dose to 500 $\mu$g resulted in somewhat lower numbers of IgG PFC and considerably decreased quantities of circulating anti-DNP. These findings may well represent a balance between tolerance induction and immunity on the one hand, and binding and subsequent clearance of anti-DNP antibodies by high levels of unmetabolized DNP-\textsuperscript{\textalpha}-GL persisting 10 days after injection on the other.

Effect of Allogeneic Cell Transfer on Primary Responses to DNP-\textsuperscript{\textalpha}-GL and DNP-OVA—

Groups of normal CAF\textsubscript{1} mice were injected intravenously with 25 $\times$ 10$^6$ normal parental A/J spleen cells, while control CAF\textsubscript{1} mice received no allogeneic cell transfer. Immediately after cell transfer, recipient and control mice were either primarily immunized with 100 $\mu$g of DNP-OVA or injected with 100 $\mu$g of DNP-\textsuperscript{\textalpha}-GL.
The results are summarized in Table III and illustrated in Fig. 4. Control mice (groups A and C) which received no allogeneic cells and were challenged with either DNP-φ-GL or DNP-OVA produced no detectable serum anti-DNP antibodies. The levels of IgM and IgG anti-DNP PFC were somewhat, but not significantly, higher in mice injected with DNP-φ-GL. The administration of allogeneic cells failed to increase the primary anti-DNP antibody response to DNP-OVA as reflected by both serum antibody levels and IgM and IgG PFC (group D). In marked contrast, the mice which received allogeneic cells and

**TABLE III**

| Protocol* | Anti-DNP antibody response† |
|-----------|-------------------------------|
| Group     | No. of A/J cells transferred | PFC/spleen (day 10) | Serum antibody |
|           |                              | IgM IgG | Day 7 | Day 10 |
| A         | None                         | 100 μg | 4,352  | 7,069  |
| B         | 25 × 10⁶                     | DNP-φ-GL | 7,764  | 40,779 |
| C         | None                         | 100 μg | 3,473  | 3,036  |
| D         | 25 × 10⁶                     | DNP-OVA | 4,390  | 3,396  |

* Groups of normal CAF₁ mice were injected intravenously with 25 × 10⁶ normal parental A/J spleen cells. Control mice received no allogeneic cells. All mice were then injected intraperitoneally with either DNP-φ-GL or DNP-OVA immediately after cell transfer. Determinations of serum antibody and splenic PFC levels were made on the days indicated after cell transfer and challenge.

† The data are expressed as geometric means of groups of four mice. A comparison of various groups yielded the following P values. Comparing group A with group B: IgM PFC, 0.50 > P > 0.40; IgG PFC, 0.025 > P > 0.02; serum antibody (day 7), P > 0.001; serum antibody (day 10), P > 0.001. There are no significant differences between group C and group D.

were injected with DNP-φ-GL developed significantly higher responses as reflected by considerably increased levels of IgG anti-DNP PFC and serum anti-DNP antibodies. There was no appreciable change in the number of IgM PFC.

**Effect of Depletion of Host T Lymphocytes on the Capacity of the Allogeneic Effect to Enhance Primary Anti-DNP Antibody Responses.**—The failure to obtain an allogeneic effect on primary responses to DNP-OVA, as shown in the preceding experiment, whereas such an effect can be reproducibly obtained with the nonimmunogen, DNP-φ-GL, raises several interesting questions concerning the nature of the phenomenon. A valid consideration is that the molecular structure of the carrier employed is critical, the phenomenon being favored perhaps by more complex molecules. However, similar experiments (not shown)
Fig. 4. The effect of allogeneic cell transfer on the primary responses to DNP-D-GL and DNP-OVA. Normal CAF₁ mice were injected intravenously with 25 × 10⁶ parental A strain spleen cells and then challenged intraperitoneally with either 100 μg of DNP-o-GL or DNP-OVA. Control CAF₁ mice, which received no allogeneic cell transfer, were challenged with either 100 μg of DNP-o-GL or 100 μg of DNP-OVA. 10 days after cell transfer and challenge, both serum anti-DNP antibody levels and IgM and IgG anti-DNP PFC were determined.

using KLH as a more complex carrier than OVA have failed to support this notion in that no enhancement of primary responses to DNP-KLH were observed as a result of allogeneic lymphoid cell transfer. Another possible explanation for these differences may be that the absence of carrier-specific T lymphocytes, which is true in the case of o-GL, is crucial for expression of a direct influence of allogeneic T cells upon the DNP-specific B cell precursors in the unprimed host lymphocyte population. This possibility was tested in a double adoptive transfer model in which host spleen cells were depleted of their T lymphocyte component by treatment with anti-θ serum and complement.

Spleen cells from normal CAF₁ donor mice were treated in vitro with either normal mouse serum (NMS) and complement or anti-θ serum and complement. These cells were mixed in vitro (on ice) with parental A/J spleen cells (ratio of 3 × 10⁶ parental cells to 20 × 10⁶ CAF₁ cells) and appropriate amounts of either DNP-o-GL or DNP-KLH and were then injected intravenously into irradiated (550 R) CAF₁ recipients. Control groups of recipient mice were injected with NMS and complement-treated CAF₁ donor cells plus antigen without allogeneic lymphoid cells. All recipients were bled and tested for splenic anti-DNP PFC 10 days after cell transfer and challenge.

The results are summarized in Table IV and illustrated in Fig. 5. As shown in Table IV, the administration of allogeneic cells markedly diminished the proliferative response of transferred syngeneic cells in irradiated CAF₁ hosts as reflected by the number of spleen cells recovered on day 10. (Compare groups B, C, E, and F with groups A and D.) The data, therefore, can be validly expressed only in terms of PFC per 10⁶ recovered cells. Control CAF₁ recipients of NMS plus complement-treated cells alone failed to develop primary responses
TABLE IV

Effect of Depletion of Host T Lymphocytes on the Capacity of the Allogeneic Effect to Enhance Primary Anti-DNP Antibody Responses

| Protocol* | Treatment of host spleen cells | No. of A/J cells transferred | Primary challenge | Spleen cells recovered per recipient | Anti-DNP antibody response (day 10) |
|-----------|-------------------------------|-----------------------------|-------------------|-------------------------------------|----------------------------------|
|           |                               |                             |                   |                                     | IgM      | IgG      |
|           |                               |                             |                   |                                     | PFC/10^6 cells | PFC/10^6 cells |
| A         | NMS + C'                      | None                        |                   |                                     | 67.4     | 28.7     | 23.5     |
| B         | NMS + C'                      | 3 × 10^6                    | 100 µg            |                                     | 31.0     | 29.0     | 69.5     |
| C         | Anti-θ + C'                   | 3 × 10^6                    | DNP-D-GL          |                                     | 20.1     | 43.6     | 111.8    |
| D         | NMS + C'                      | None                        |                   |                                     | 78.0     | 42.1     | 42.9     |
| E         | NMS + C'                      | 3 × 10^6                    | 100 µg            |                                     | 28.4     | 31.4     | 39.5     |
| F         | Anti-θ + C'                   | 3 × 10^6                    | DNP-KLH           |                                     | 20.0     | 13.7     | 14.8     |

* Spleen cell suspensions from normal CAF1 mice, treated with either NMS and complement or anti-θ serum and complement, were injected intravenously into 550 R-irradiated syngeneic recipients, with or without parental A/J spleen cells, and with either 100 µg of DNP-D-GL or 100 µg of DNP-KLH. Determination of splenic PFC levels were made 10 days after cell transfer and challenge.

† The data are expressed as geometric means of groups of five mice. A comparison of various groups yielded the following P values. For IgM PFC: comparison of group A with group B yielded 0.98 > P > 0.975; group A with group C, 0.30 > P > 0.20; group D with group E, 0.40 > P > 0.30; group D with group F, 0.01 > P > 0.005. For IgG PFC: comparison of group A with group B yielded 0.01 > P > 0.005; group A with group C, 0.05 > P > 0.025; group D with group E, 0.80 > P > 0.70; group D with group F, 0.005 > P > 0.001.

to DNP-D-GL (group A) and only barely appreciable increases in anti-DNP PFC levels with DNP-KLH (group D). The administration of parental allogeneic lymphoid cells resulted in significant increases in primary anti-DNP PFC responses, particularly of the IgG class, of mice challenged with DNP-D-GL. This was true irrespective of whether the original donor inoculum of CAF1 cells had been treated with NMS and complement (group B) or anti-θ serum plus complement (group D). In contrast, comparable recipient mice which were challenged with DNP-KLH (groups E and F) failed to display any increase in anti-DNP PFC. In fact, the PFC levels obtained in these groups, particularly in the case of recipients of anti-θ serum-treated CAF1 donor cells, were lower than those manifested by control mice in group D, probably reflecting the loss of KLH-specific T lymphocytes.

Capacity of the Allogeneic Effect to Enhance the Primary Responses to Both DNP-D-GL and DNP-L-GL.—The failure of the allogeneic effect to induce an enhanced primary response to DNP-KLH after depletion of host T lymphocytes argues strongly that it is not merely the absence of carrier-specific T lymphocytes which enables this effect to be shown so well with DNP-D-GL, but rather
some intrinsic feature of the carrier molecule itself. To test this hypothesis, the following study was performed comparing the capacity of allogeneic cells to enhance the primary response to both DNP-D-GL and DNP-L-GL. DNP-L-GL is a DNP conjugate of the random copolymer of L-glutamic acid and L-lysine. Whereas DNP-D-GL is a nonmetabolizable molecule which easily induces tolerance over a wide dose range, DNP-L-GL is a readily metabolizable molecule which, in inbred mice, is "nonimmunogenic" at all doses presumably because of a lack of carrier-specific helper T lymphocytes, and which induces tolerance only in a very restricted dose range (Katz, D. H., T. Hamaoka, and B. Benacerraf, unpublished observations).

Normal CAF1 mice were injected intravenously with 50 × 10⁶ parental A/J spleen cells, while control CAF1 mice received no allogeneic cell transfer. Immediately after cell transfer, control and recipient mice were injected intraperitoneally with either 100 µg of DNP-D-GL or 100 µg of DNP-L-GL. All mice were killed 10 days later for determinations of splenic anti-DNP PFC.

The results are shown in Table V. Control mice (groups A and C) which received no allogeneic cells and were challenged with either DNP-D-GL or DNP-L-GL displayed only background levels of both IgM and IgG anti-DNP...
TABLE V
Capacity of the Allogeneic Effect to Enhance the Primary Responses to DNP-n-GL and DNP-L-GL

| Group | No. A/J cells transferred | Primary challenge | IgM | IgG |
|-------|---------------------------|-------------------|-----|-----|
|       |                           |                   | PFC/spleen | PFC/spleen |
| A     | None                      | 100 µg            | 1,263 | 2,326 |
| B     | 50 × 10^6                 | DNP-n-GL          | 5,910 | 12,254 |
| C     | None                      | 100 µg            | 2,030 | 1,332 |
| D     | 50 × 10^6                 | DNP-L-GL          | 3,143 | 4,789 |

* Normal CAF1 mice were injected intravenously with 50 × 10^6 parental A/J spleen cells. CAF1 mice which received no cell transfer served as controls. Mice from both groups were injected with either 100 µg of DNP-n-GL or 100 µg of DNP-L-GL shortly after cell transfer. Determination of splenic PFC levels were made 10 days after cell transfer and challenge.

† The data are expressed as geometric means of groups of five mice. A comparison of various groups yielded the following P values. For IgM PFC: comparison of group A with group B yielded 0.01 > P > 0.005; group C with group D, 0.20 > P > 0.10. For IgG PFC: comparison of group A with group B yielded 0.001 > P; group C with group D, 0.005 > P > 0.001.

On the other hand, recipients of allogeneic lymphoid cells manifested increased numbers of splenic PFC when challenged with either DNP-n-GL or DNP-L-GL. As seen previously, this enhancement of the primary response was particularly marked in PFC of the IgG antibody class. The fact that the magnitude of the response was greater after challenge with DNP-n-GL than with DNP-L-GL most probably reflects the more rapid metabolism of DNP-L-GL and its subsequent removal from the system.

DISCUSSION

Since the demonstration that cooperation between T and B lymphocytes is required for antibody production to the vast majority of antigens (19–21), considerable attention has been focused on elucidating the nature of these cellular interactions and the details of the control mechanisms implicit in them (for review, see reference 22). The allogeneic effect phenomenon has served as one highly useful model for investigating the regulatory influences of T lymphocytes on B cell responses to antigen (reviewed in reference 7). Indeed, this phenomenon has provided a forceful argument for the concept of the expression of T cell function via release of nonspecific mediators active in the process of B cell triggering by antigen (7, 22).

In our previous studies in this system, it was quite clear that the responding B cell population must be primed before the allogeneic cell transfer in order for the phenomenon to occur (1–4). Attempts to demonstrate enhanced primary responses to typical antigens such as DNP-OVA or DNP-KLH not only failed,
but frequently resulted in marked suppression of the primary response. In the present study, we have altered our previous approaches to investigating the influence of the allogeneic effect on primary antibody responses by utilizing the nonimmunogen, DNP-D-GL. As pointed out in the introduction, this conjugate has several unique features. Particularly important is the capacity of DNP-D-GL to induce a profound state of DNP-specific tolerance in both normal and DNP-primed animals (2, 10). However, in the presence of the allogeneic effect, this same molecule provides a positive triggering signal (2, 4). This represents, perhaps, the most relevant model of the direct influence of activated T cells on B lymphocytes. Thus, B cells exposed to DNP-D-GL in the absence of activated T cells and/or their product(s) are rapidly rendered tolerant, whereas exposure to DNP-D-GL in the presence of T cell function results in DNP-specific immunity. These contrasting results, clearly determined by the presence or absence of T cell activity, led us to consider that the activated T cells and/or their product(s) which play such a crucial role in the triggering of primed B cells exposed to DNP-D-GL, may influence as well the response of unprimed B lymphocytes to this normally tolerogenic compound.

Indeed, the observations reported here illustrate that the presence of non-specific T cell function generated during the allogeneic effect alters the ultimate response of unprimed B lymphocytes to DNP-D-GL. Thus, normal CAF1 mice, which ordinarily fail to develop antibody responses to this compound, manifested significant primary anti-DNP responses when DNP-D-GL was administered in conjunction with parental A/J donor spleen cells. The primary responses obtained in this way were particularly notable in that they were predominantly of the IgG antibody class. The capacity to induce such an effect, as shown here, is determined by the concomitant influences exerted by variables such as timing of the allogeneic cell transfer in relation to primary challenge, number of allogeneic cells employed, and the dose of DNP-D-GL administered. However, in contrast to relatively greater restrictions in this regard insofar as the allogeneic effect on secondary antibody responses in mice (4), these variables operate over a considerably wider range to permit expression of the phenomenon in primary responses to DNP-D-GL. Hence, the effect was observed in the present studies when allogeneic cells ranging in number from $10^7$ to $10^8$ per recipient were administered over intervals from 5 days before to simultaneously with primary inoculation of various doses of DNP-D-GL. The peak effect appeared to occur when $50 \times 10^6$ parental spleen cells were administered on the same day as primary challenge with 100 mg of DNP-D-GL. Moreover, it has been essential to examine antibody production at the cellular level, i.e. anti-DNP splenic PFC, as well as the level of serum antibody in order to appreciate the absolute magnitude of the phenomenon. This is necessitated by the fact that DNP-D-GL, itself nonmetabolizable, tends to remain in the system with the capacity to bind to and result in the subsequent clearance of circulating anti-DNP antibody molecules.
These findings emphatically demonstrate that what is normally a signal for DNP-specific tolerance can be converted to a signal for DNP-specific immunity in the appropriate atmosphere of T cell activation and possible mediator release. The fact that the antibody response obtained under such circumstances is predominantly of the IgG class underscores the importance of T cell regulatory influences on the class shift from IgM to IgG in humoral immune responses (22, 23). The latter point was also nicely illustrated in the recent experiments of Ordal and Grumet (8) in genetic nonresponder mice which are believed to lack functional T cells with specificity for (T, G)-A-L (24). In their system, genetic nonresponders normally develop primary antibody responses to aqueous (T, G)-A-L which are restricted to the IgM antibody class. However, induction of an appropriately timed GVH reaction permitted such nonresponder mice to develop primary responses to (T, G)-A-L of both IgM and IgG antibody classes (8). In this sense, our present results fully corroborate their important observation.

An obvious point of similarity between the present studies and those of Ordal and Grumet cited above (8) is the fact that in both systems the allogeneic effect exerts demonstrable influences on primary antibody responses to substances for which, in the animals employed, no helper T cell function exists. More recently, a similar phenomenon of enhanced antibody production has been found in primary responses to the thymus-independent type III pneumococcal polysaccharide (SIII) in mice undergoing a GVH reaction. On the other hand, repeated attempts to obtain any appreciable allogeneic effect on primary antibody responses to immunogenic conjugates such as DNP-OVA or DNP-KLH have failed in the present experiments as well as in studies reported previously (1-3, 7). Taken collectively, these various findings indicate that the capacity of the allogeneic effect to exert any enhancing influence on the responses of unprimed B lymphocytes to specific molecules is largely determined by the nature of the molecule employed.

As an explanation for the role of molecular structure, one may consider several possible factors. First, since all of the aforementioned molecules have been either inherently or consequently operating in the absence of specific functional T cells, it is conceivable that the very presence of antigen-specific T cell function may interfere with the expression of nonspecific T cell participation (allogeneic effect). This concept is frankly difficult to reconcile with the many observations made in studies of the allogeneic effect in secondary responses (7) unless it is accepted that fundamental differences between primed vs. unprimed B lymphocytes are critical in this regard. Nonetheless, based on this reasoning, we examined the possibility by using adoptively transferred unprimed spleen cell populations which had been depleted of the T lymphocyte

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component by treatment with anti-θ serum plus complement. Under these circumstances, it was still possible to obtain an allogeneic effect on primary antibody responses to DNP-d-GL. Conversely, such T cell-depleted spleen cell populations failed to develop primary responses to DNP-KLH irrespective of whether or not allogeneic cells were concomitantly administered. This observation argues against the possibility that the presence of antigen-specific functional T cells, or, for that matter, any T cells results in an interference in the expression of the allogeneic effect on the primary response.

A second possibility to explain the observations concerns the inherent molecular structure of the substances employed. The common feature of DNP-d-GL, DNP-l-GL, (T,G)-A--L, and SIII is their more or less repetitive determinant structure. This type of determinant arrangement appears to favor, under certain circumstances, the transduction of immunogenic signals to at least some B lymphocytes in the absence of T cell function (22). The antibody responses obtained with these and other “thymus-independent” antigens are, however, restricted to the IgM class which indicates that despite their capacity to trigger specific B cell precursors of this class, the switch to IgG requires the additional participation of T lymphocytes regardless of the molecular structure of the antigen. The fact that the presence of T cell function as provided by the allogeneic effect permits the development of IgG antibody responses to DNP-d-GL or DNP-l-GL as shown herein and to (T,G)-A--L in nonresponder mice (8) demonstrates not only the critical role of T cells in the IgM to IgG switch but also that these molecules are not in themselves prohibitive to a triggering event in IgG precursor cells. Clearly, we have no definitive data as to why the unique structure of certain molecules permits triggering of IgM antibody responses in the absence of T cell participation. Nor do we know why these very same types of molecules elicit enhanced primary antibody responses, of the IgG class, in the presence of the allogeneic effect whereas typical multideterminant molecules such as DNP-KLH or DNP-OVA do not. What seems clear, however, is that the molecular features which dictate thymus independence are also essential for expression of the allogeneic effect on antibody responses in unprimed cell populations.

A final consideration must be given to the different conditions under which allogeneic lymphocytes are able to affect unprimed and primed B cell populations. It is clear that very critical differences must exist since primed B lymphocytes have been amply shown to manifest enhanced responsiveness to all antigens as a result of the allogeneic effect. The responsiveness of such lymphocytes can also be suppressed under the appropriate conditions of the allogeneic effect (4, 7). In contrast, unprimed B lymphocytes, although often suppressed by the induction of a GVH reaction, are readily susceptible to immediate enhancing effects of this phenomenon, only with respect to a selected class of antigen molecules. Thus, insofar as the triggering signal for differentiation into antibody-secreting cells is concerned, maturational changes in B lymphocytes
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1006 occasioned by interactions with antigen and T lymphocytes appear to impart qualitative differences in the reactivities of such cells not only to subsequent exposure to antigen but to T cell influences as well. By this reasoning unprimed B lymphocytes may possess a more restricted threshold of reactivity which, for typical antigens, places great limitations on their capacity to be influenced by T cell activity during early stages of immune induction. On the other hand, structurally unique molecules as exemplified in these studies by DNP-D-GL may result in a type of cooperative receptor-binding event which drastically changes the reactivity threshold of specific B lymphocytes and, irrespective of the state of priming, results in a higher level of susceptibility of such cells to the enhancing influences of activated T cells. Whether the unique antigenic structural requirements for expression of the allogeneic effect in vivo on unprimed vs. primed cell populations can be explained simply on the basis of maturation of a specific primed B lymphocyte population to progressively higher average receptor affinity or, rather, by certain undefined consequences of the allogeneic cell interactions merit further investigation.

Although no allogeneic effect has been obtained in terms of immediate antibody production to typical antigens in these studies, it is not inconceivable and is perhaps quite likely that some enhancing influences on priming and subsequent expression of specific memory may occur in such circumstances. Studies designed to delineate this possibility and the predominant host cell(s) affected, i.e. B or T lymphocyte (or both), are under way.

SUMMARY

The allogeneic effect has been shown to replace the requirement for carrier-specific helper thymus-derived (T) lymphocytes in secondary antihapten antibody responses in guinea pigs or mice. Attempts to enhance primary antibody responses to either 2,4-dinitrophenyl (DNP)-keyhole limpet hemocyanin (KLH) or DNP-ovalbumin (OVA) by the allogeneic effect have failed, and frequently result in suppression. However, the present studies have demonstrated a clear allogeneic effect on primary anti-DNP responses to a DNP-conjugate of the copolymer of D-glutamic acid and D-lysine, DNP-D-GL. This compound, for which no carrier-specific helper T cells exist, normally induces a state of DNP-specific tolerance in the doses employed. However, normal (BALB/c × A/J)F1 recipients developed primary anti-DNP antibody responses, and of the IgG class, when DNP-D-GL was administered shortly after the transfer of allogeneic parental A strain lymphoid cells. To test the possibility that the presence of KLH-specific T lymphocytes might inhibit the expression of the allogeneic effect on the primary response to DNP-KLH, studies were undertaken using T cell-depleted spleen cells. In this model, the allogeneic effect again enhanced the primary response to DNP-D-GL, but still failed to enhance the primary response to DNP-KLH. These studies indicate that the structure of the molecule employed and its specific interaction with the bone
marrow-derived (B) cell membrane may be critical in the capacity of primed and unprimed B cells to be influenced by the allogeneic effect.

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