

## THE ALLOGENEIC EFFECT IN INBRED MICE

### II. ESTABLISHMENT OF THE CELLULAR INTERACTIONS REQUIRED FOR ENHANCEMENT OF ANTIBODY PRODUCTION BY THE GRAFT-*VERSUS*-HOST REACTION\*

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A critical question raised by the initial observations in guinea pigs (1) and in the studies in mice presented in the preceding paper (2) is that of the precise role of the host T cell in the mediation of the allogeneic effect on antibody production. Thus, the phenomenon requires the existence of an active graft-*versus*-host (GVH)<sup>1</sup> reaction in which the primed B lymphocytes are those of the host cell population. The occurrence of a host rejection reaction is not only not required, but also appears to play little, if any, role (1, 3). This suggests that, by direct action on primed B cells, the allogeneic effect obviates entirely the need for the interaction between isologous T and B lymphocytes specific for the challenging conjugate, or, alternatively, enhances the functional effectiveness of the small number of isologous T cells specific for the second carrier.

To approach this issue directly requires a system in which both cell populations, i.e. the 2,4-dinitrophenyl (DNP)-primed population and a population of allogeneic lymphoid cells, are accessible to experimental manipulation such that the T lymphocytes of one or the other can be selectively removed. In the present study, we describe conditions for the elicitation of an allogeneic effect on the adoptive transfer secondary anti-DNP antibody response in mice. Utilizing such a model, we have established that the allogeneic effect on antibody production can operate on a population of primed B lymphocytes which have been depleted of their isologous T lymphocytes.

#### *Materials and Methods*

The proteins and hapten-carrier conjugates were identical to those described in the preceding paper (2).

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<sup>1</sup> *Abbreviations used in this paper:* BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; D-GL, copolymer of D-glutamic acid and D-lysine; DNP, 2,4-dinitrophenyl; GVH, graft-*versus*-host; KLH, keyhole limpet hemocyanin; MEM, Eagle's minimum essential medium.

*Animals.*—Mice of the inbred strains BALB/c, C57BL/6N, AKR, and C3H/HeN were obtained from the Jackson Laboratory, Bar Harbor, Maine. Certain BALB/c mice were also obtained from West Seneca Laboratories, Buffalo, N.Y.

*Adoptive Transfer System.*—The basic scheme followed is diagrammatically outlined in Fig. 1. Male BALB/c mice, 8–12 wk of age, received primary immunization with 100  $\mu$ g of DNP<sub>9</sub>-keyhole limpet hemocyanin (KLH) emulsified in complete Freund's adjuvant (CFA) intraperitoneally. 2 wk later, these DNP-KLH-primed mice were killed and their spleens re-

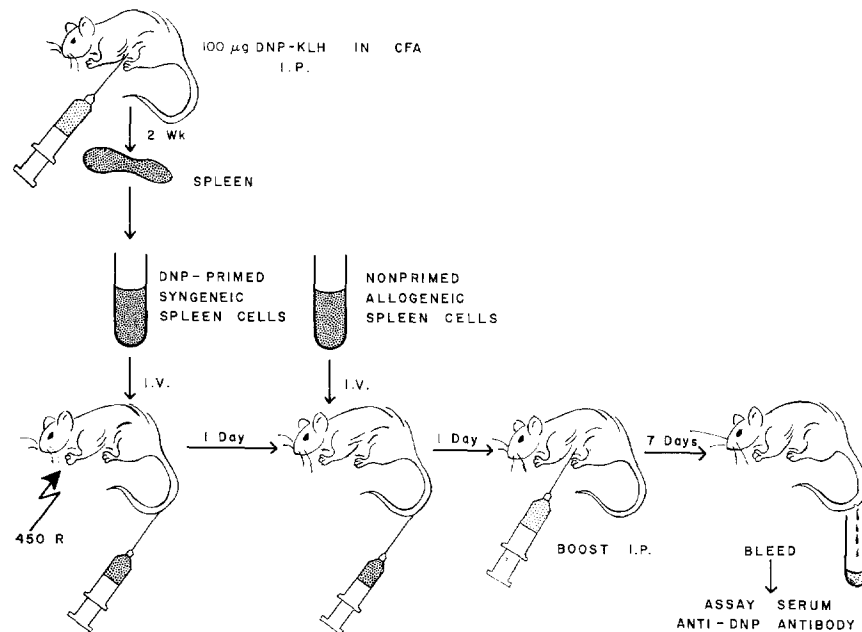


FIG. 1. Model II: transfer of DNP-primed syngeneic and nonprimed allogeneic lymphoid cells into irradiated host. Experimental protocol for elicitation of the allogeneic effect on the adoptive transfer secondary anti-DNP response. See Materials and Methods for explanation.

moved. Single cell suspensions in minimal essential medium (MEM) (Eagle) were prepared, washed, and transferred intravenously to syngeneic, irradiated (450 R) BALB/c recipients. Preliminary experiments established that a dose of  $25 \times 10^6$  DNP-KLH-primed nucleated cells was optimal for conditions of these studies and was therefore used throughout. 24 hr after transfer of the DNP-KLH-primed cells, a second intravenous cell transfer consisting of varying numbers of splenic lymphoid cells from nonimmune allogeneic C57BL/6N donors was performed in certain recipient mice. Control recipients did not receive an allogeneic cell transfer. 1 day after the second cell transfer mice were either subjected to a secondary challenge intraperitoneally with an aqueous DNP-carrier conjugate or were not secondarily immunized. All mice were bled 7 days after challenge from the retroorbital plexus and serum anti-DNP antibody levels were determined as described previously (4).

*Preparation of Anti- $\theta$  Serum.*—Anti- $\theta$ C3H serum was prepared according to the general scheme described by Reif and Allen (5). Briefly, thymocytes were obtained from 4–6-wk old C3H/HeN mice and injected intraperitoneally at a dose of  $100 \times 10^6$  cells into individual AKR male mice. 1 and 2 wk after the last of six consecutive weekly immunizations, all mice

were bled and the sera pooled. 1 wk thereafter all mice received a final injection of C3H thymocytes and were bled again 7 days later.

*Determination of Anti- $\theta$  Serum Cytotoxicity.*—Cytotoxicity of the heat-inactivated (56°C for 30 min) anti- $\theta$  sera was measured by a method utilizing chromium-51 release as the index of cytolysis in the presence of appropriately diluted guinea pig complement. This procedure has been described in detail by Stobo et al. (6). The anti- $\theta$  sera employed lysed 90–95% of thymocytes (C3H or BALB/c) at a 1:100 dilution and 30–40% of splenic lymphocytes at a 1:9–1:27 dilution.

*Anti- $\theta$  Serum Treatment of DNP-KLH-Primed Splenic Lymphocytes.*—Single spleen cell suspensions were treated with anti- $\theta$  serum and complement as follows: BALB/c spleen cells were suspended in 10% calf serum-supplemented MEM at a concentration of  $100 \times 10^6$  cells per 0.5 ml. 0.3–0.5 ml of heat-inactivated anti- $\theta$ C3H was added to each such sample and the mixture incubated at 4°C for 30 min, washed, and then resuspended in 0.5 ml of medium. 1.0 ml of guinea pig complement (diluted 1:2 in medium) was added to each portion and the mixture incubated for an additional 40 min at 37°C. After thorough washing, remaining cells were resuspended in the appropriate volume of MEM and then transferred to irradiated recipients intravenously. Viability of recovered cells was 89–97% by dye exclusion (trypan blue) and the usual total recovery of viable cells after such treatment was in the range of 55%.

#### RESULTS

*The Allogeneic Effect on the Adoptive Transfer Secondary Anti-DNP Response.*—Following the scheme outlined in Materials and Methods (Fig. 1),  $25 \times 10^6$  spleen cells from DNP-KLH-primed BALB/c donor mice were injected intravenously into irradiated syngeneic BALB/c recipients. 24 hr later, groups of these recipients were injected with varying numbers of allogeneic cells from nonimmunized C57BL donors; groups of control mice received no allogeneic cells. 1 day after the second cell transfer (48 hr after initial adoptive transfer) the recipients were challenged with 10  $\mu$ g of DNP-KLH or 50  $\mu$ g of DNP bovine gamma globulin (BGG) intraperitoneally, or not secondarily challenged. All mice were bled 7 days after secondary challenge.

The results are illustrated graphically in Fig. 2. Recipients of DNP-KLH-primed syngeneic cells but no additional allogeneic cells manifested sharp adoptive secondary anti-DNP responses to the homologous antigen, DNP-KLH, but failed to produce appreciable quantities of antibody to a challenge with DNP-BGG. In contrast, recipients of DNP-primed cells which were also injected with allogeneic cells developed secondary responses to DNP-BGG that were related in magnitude to the number of allogeneic cells administered. Thus, the dose of  $2.5 \times 10^6$  cells (an allogeneic-to-primed cell ratio of 1:10) elicited the optimal effect.  $5 \times 10^6$  allogeneic cells resulted in a significant enhancement over control responses, though not as great as that obtained with  $2.5 \times 10^6$  cells, while  $1 \times 10^6$  cells produced only a slight increase in response over controls. All mice in an experimental group which received  $10 \times 10^6$  allogeneic cells died before the 7 day bleeding, presumably as a result of the intense GVH reaction.

*Elicitation of the Allogeneic Effect on DNP-Primed Cells Depleted of Isologous T Lymphocytes.*—With the demonstration in the preceding experiment that an allogeneic effect could be elicited in a suitable adoptive transfer model, a study could be made of the requirement of host T cells, i.e. those T lymphocytes isolo-

gous to the DNP-primed B cells in the system, for the expression of the phenomenon. In order to accomplish this, experiments were performed, using the

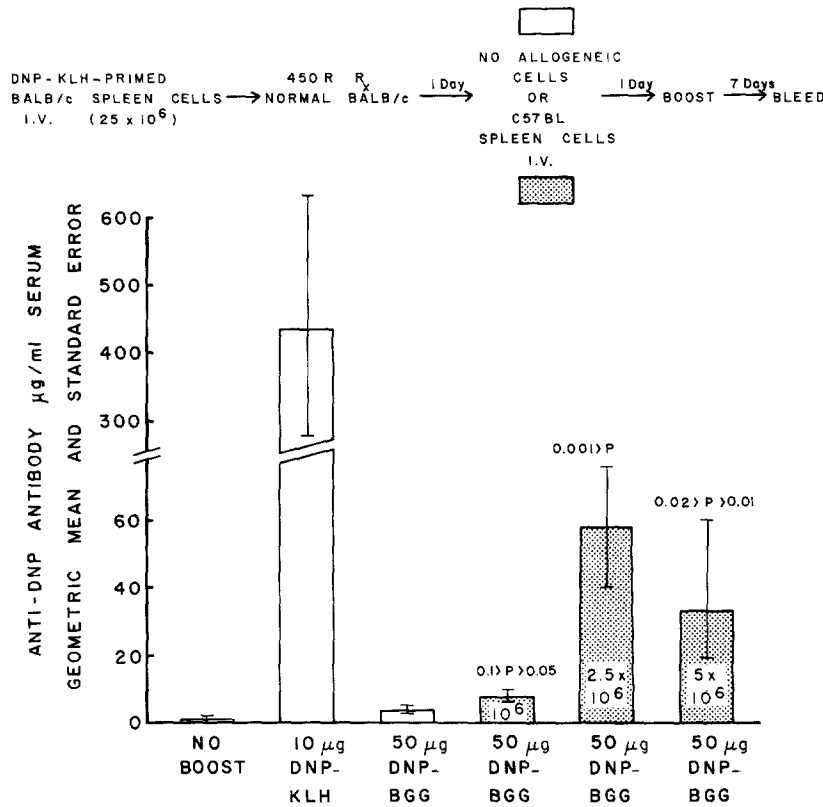


FIG. 2. The allogeneic effect on the adoptive transfer secondary anti-DNP antibody response.  $25 \times 10^6$  spleen cells from BALB/c donor mice, primed 2 wk previously with  $100 \mu\text{g}$  of DNP-KLH emulsified in CFA, were injected intravenously into irradiated syngeneic BALB/c recipients. 24 hr later, groups of these recipients were injected intravenously with varying numbers of allogeneic spleen cells from nonimmunized C57BL donors; groups of control mice (open bars) did not receive allogeneic cells. 1 day after the second cell transfer, the recipients were either not challenged or secondarily challenged with  $10 \mu\text{g}$  of DNP-KLH or  $50 \mu\text{g}$  of DNP-BGG intraperitoneally. The geometric mean levels of serum anti-DNP antibody of groups of five mice bled 7 days after secondary challenge are illustrated. A comparison of the recipients of allogeneic cells secondarily challenged with DNP-BGG with the control group also challenged with DNP-BGG yielded  $P$  values as shown.

optimal conditions established in the preceding experiment, in which a comparison was made of the capacity to elicit the allogeneic effect on DNP-primed cells which had or had not been depleted of their isologous T lymphocytes.

Spleen cells from DNP-KLH-primed BALB/c were either treated *in vitro* with anti- $\theta$  serum plus complement or not treated and then injected intravenously into irradiated syn-

genic BALB/c recipients. Each recipient was injected with  $25 \times 10^6$  untreated or anti- $\theta$ -treated cells. 24 hr after the first cell transfer, groups of recipients of each cell type (untreated or anti- $\theta$  treated) were injected intravenously with  $2.5 \times 10^6$  allogeneic C57BL spleen cells while corresponding control groups received no allogeneic cell transfer. 1 day later, mice were secondarily challenged with either 10  $\mu$ g of DNP-KLH or 50  $\mu$ g of DNP-BGG intraperitoneally, or not challenged. All mice were bled 7 days after secondary immunization.

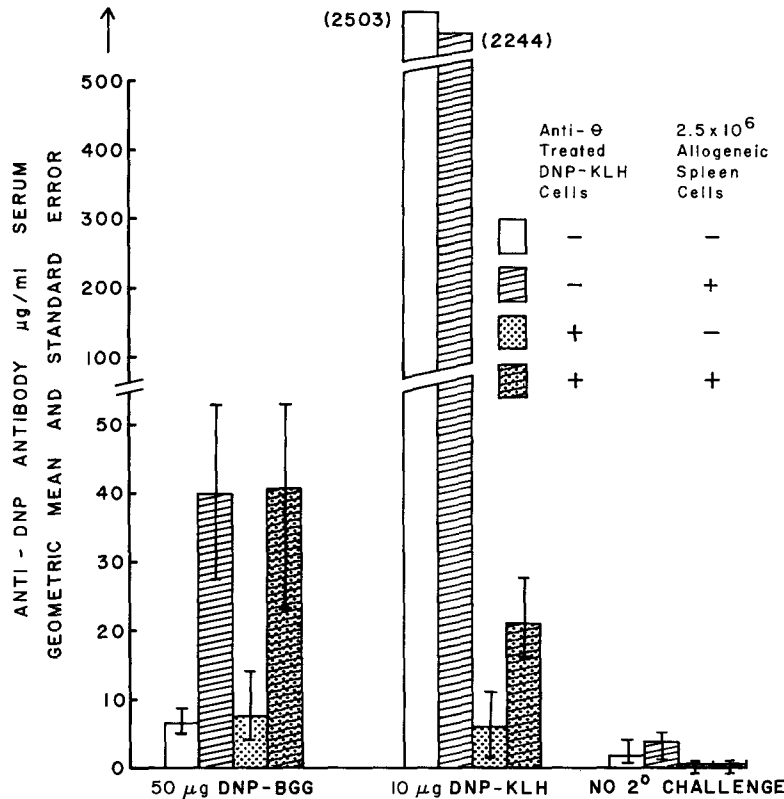


FIG. 3. Elicitation of the allogeneic effect on DNP-KLH-primed cells depleted of isologous T lymphocytes. Spleen cells from BALB/c donors, primed 2 wk earlier with 100  $\mu$ g of DNP-KLH in CFA, were either treated in vitro with anti- $\theta$  serum plus complement or not treated and then injected intravenously ( $25 \times 10^6$  cells/recipient) into irradiated syngeneic BALB/c recipients. 24 hr later, groups of recipients of each cell type (untreated or anti- $\theta$  treated) were injected intravenously with  $2.5 \times 10^6$  allogeneic C57BL spleen cells. Corresponding control groups received no allogeneic cell transfer. 1 day after the second cell transfer, mice were secondarily challenged with either 10  $\mu$ g of DNP-KLH or 50  $\mu$ g of DNP-BGG intraperitoneally, or not challenged. The geometric mean levels of serum anti-DNP antibody of groups of five mice bled 7 days after secondary challenge are illustrated. Statistical comparisons of the recipients of DNP-KLH cells challenged with DNP-BGG gave the following results. Comparison of allogeneic cell recipients with controls yielded a  $P$  value of  $0.10 > P > 0.05$  for anti- $\theta$ -treated cells. Comparison of allogeneic cell recipients of anti- $\theta$ -treated cells secondarily challenged with DNP-BGG with recipients of anti- $\theta$ -treated cells not given allogeneic cells and secondarily challenged with DNP-KLH yielded a  $P$  value of  $0.05 > P > 0.025$ .

As depicted graphically in Fig. 3, recipients of syngeneic DNP-primed spleen cells which were not subjected to anti- $\theta$  treatment produced, as expected, high levels of anti-DNP antibody after secondary challenge with DNP-KLH irrespective of whether or not they received a second transfer of allogeneic cells. On the other hand, recipients of untreated DNP-primed cells alone failed to respond to DNP-BGG, whereas a significantly enhanced anti-DNP response to DNP-BGG was obtained in those recipients also injected with allogeneic lymphoid cells. Treatment of the DNP-primed cells with anti- $\theta$  plus complement in vitro before adoptive transfer abolished the secondary response to DNP-KLH in those mice which did not also receive allogeneic cells. In contrast, recipients of anti- $\theta$ -treated DNP-primed cells plus allogeneic cells displayed clearly enhanced anti-DNP antibody responses both to DNP-KLH and DNP-BGG. The highest responses were obtained after secondary challenge to DNP-BGG and it is particularly noteworthy that the magnitude of the allogeneic effect on the anti- $\theta$ -treated population in response to the latter conjugate was comparable in magnitude to that obtained with the untreated DNP-primed cells. The finding that the responses to DNP-BGG were greater than those obtained with the homologous antigen, DNP-KLH, in these circumstances probably reflects the fact that the molar concentration of DNP administered was roughly 30-fold greater in the case of DNP-BGG as compared with DNP-KLH. Comparable observations were obtained in an essentially identical experiment in which control DNP-KLH-primed cells were treated with normal mouse serum plus complement in vitro before adoptive transfer. These results strongly indicate, therefore, that the participation of host (or isologous) T lymphocytes is not required for the expression of the allogeneic effect phenomenon on antibody production.

#### DISCUSSION

It is now well established that under appropriate conditions the GVH reaction may greatly enhance antibody production to a variety of antigens both in vivo (1-3, 7, 7a) and in vitro (7a, 8, 9). In antibody responses to hapten-carrier conjugates, evidence has been presented that the allogeneic effect replaces the requirement for carrier-specific helper T cells normally participating in such immune reactions (1-3, 7, 7a). This latter point could result from the allogeneic effect acting either: (a) to heighten stimulation and/or increase the functional efficiency of the small numbers of helper T cells specific for the carrier in question that presumably exist in the virgin population; or (b) to create a critical alteration, either in milieu or in the cell itself, that permits the specific B lymphocyte to respond to the antigenic stimulus, with or without the participation of isologous T lymphocytes.

In its fullest consideration, the allogeneic effect may involve a number of cell interactions between and among donor and host lymphocytes. These potential interactions are illustrated schematically in Fig. 4 in which cell populations

are identified as unprimed and DNP primed (analogous, respectively, to donor and host cells in the classical *in vivo* model) to facilitate adaptation of the scheme to adoptive transfer or *in vitro* systems. Disregarding the theoretical interaction between the two B cell populations, there are five interactions potentially occurring after mixture of two allogeneic populations: I, II, and IV involve mixed lymphocyte interactions based on surface histocompatibility antigens, whereas III and V represent the interactions between isologous T and B lymphocytes. It is now possible to dissect this scheme in the context of the allogeneic effect phenomenon on antibody production as follows.

Firstly, we can essentially disregard the interactions IV and V in this scheme

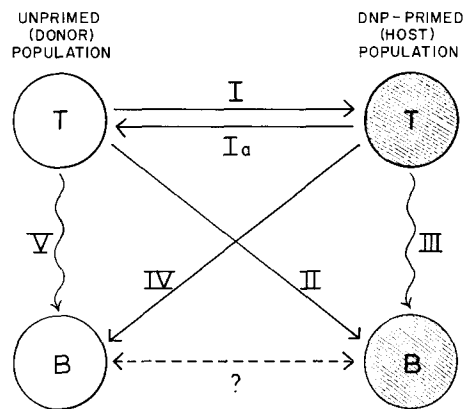


FIG. 4. Cellular interactions in the allogeneic effect I. See text of Discussion for explanation.

since it has been previously demonstrated that antibody is produced solely by B lymphocytes of the primed cell population (3). Thus, although IV and V may and probably do occur, their contribution to the expression of the allogeneic effect is negligible. Secondly, the available evidence argues against a significant, if any, role for interaction Ia, i.e. the rejection reaction of T lymphocytes of the unprimed (donor) population by T lymphocytes isologous to the primed B cell population. This conclusion is based on the following observations: (a) the transfer of immunologically incompetent allogeneic lymphoid cells, irrespective of numbers, fails to elicit enhanced antibody production in primed hosts which are capable of recognizing and rejecting such cells (1); (b) transfer of parental lymphoid cells elicits an allogeneic effect in primed F<sub>1</sub> hybrid recipients which are genetically incapable of mounting a reciprocal rejection reaction (3); (c) no enhanced antibody production occurs after transfer of primed lymphoid cells into allogeneic irradiated recipients, a circumstance in which the transferred cells react against a host that has been rendered incapable of developing a rejection response (2).

Having eliminated considerations of the interactions above, we are left with

the remaining possibilities as represented schematically in Fig. 5. The allogeneic effect, in addition to enhancing antibody production, may also have a similar influence on cell-mediated immune responses. Evidence for this is derived from recent studies in which the highly fatal course of acute lymphocytic leukemia ( $L_2C$ ) in inbred strain 2 guinea pigs was significantly altered after induction of a transient GVH reaction (10, 11). Since detailed studies of specific immunity to this particular leukemia have demonstrated that it is predominantly, if not solely, a cell-mediated immunity (12), it is possible that the protection observed during the allogeneic effect reflects enhancement of cell-mediated immunity. Moreover, recent experiments utilizing a more direct approach

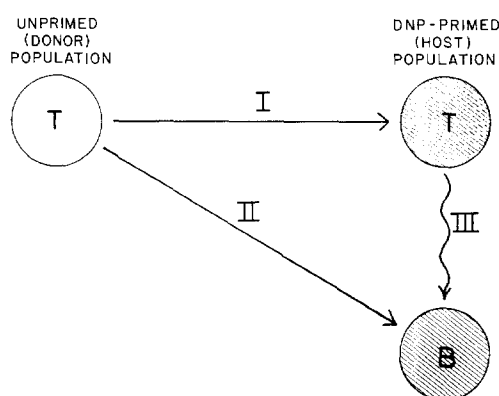


FIG. 5. Cellular interactions in the allogeneic effect II. See text of Discussion for explanation.

to this question have demonstrated that delayed hypersensitivity reactions in guinea pigs can be significantly augmented by the induction of transient GVH reactions.<sup>2</sup> Hence, interaction I between donor and host T lymphocytes appears to have an enhancing effect on subsequent functions of the latter. Although this implies, on the surface, that the same mechanism may operate to increase the antibody responses of the primed B lymphocyte population, i.e. by enhancing in some way the interaction III between isologous T and B lymphocytes of the primed cell population, the results presented in this paper as well as earlier observations in guinea pigs and mice (1-3) as discussed above are not consistent with this interpretation.

In the present study, we have shown that, indeed, depletion of the isologous T lymphocytes from the primed cell population does not prevent the expression of the allogeneic effect on antibody responses of the B lymphocyte population. The efficacy of T cell depletion by anti- $\theta$  serum and complement in these experiments is reflected by the abrogation of the anti-DNP response of such

<sup>2</sup> Elgenbein, G., W. E. Paul, and I. Green. Unpublished observations.



cells to the homologous DNP-KLH conjugate. Moreover, it is most likely that any T cells bearing  $\theta$  determinants in low density and thereby escaping complement-induced cytolysis were rapidly removed by phagocytic cells of the reticuloendothelial system in vivo after intravenous transfer (C. S. Henney and J. D. Stobo, personal communication). A similar result has been recently reported by Kreth and Williamson (13). These observations are, furthermore, consistent with and explain the ability to elicit secondary anti-DNP responses with DNP-copolymer of D-glutamic acid and D-lysine (D-GL). As discussed earlier (7), it is presumed, and there is evidence for,<sup>3</sup> the notion that D-GL does not stimulate an effective T cell helper function. It appears, therefore, that with respect to the allogeneic effect on antibody production, interactions I and III of Fig. 5 play a negligible role.

This leaves one remaining interaction (II), namely that of the reaction of the foreign T lymphocyte against the primed B lymphocyte, to consider. Indeed, as we have shown here, it is this interaction which is the most crucial in the phenomenon. Since the precise nature of cellular events during this interaction is undefined, we can only speculate as to how it might result in increased antibody production: (a) direct membrane-membrane interaction between the two cells occurring, of course, under limiting circumstances in which the B lymphocyte is not killed may stimulate increased proliferation and/or metabolic activity of the latter. (b) Release of nonspecific stimulatory factors from the T lymphocyte in conditions favored by the intimate spatial relationship may markedly increase the magnitude and/or efficiency of the B cell response to antigen. (c) A concomitant effect mediated by the occurrence of both direct membrane-membrane contact and the release of T cell-produced factors capable of influencing the response of B cells to antigen may occur.

As discussed in the preceding paper (2) and at length elsewhere (7a, 14),<sup>3</sup> it should be emphasized that, in circumstances of potent T cell activation such as the allogeneic effect, antibody production may be diminished rather than increased as a consequence of, perhaps, the very same mechanism(s) enumerated above depending on the extent of the interaction. It is possible that elucidation of the above alternatives in the allogeneic effect phenomenon may provide important insight into the critical events of cell stimulation by antigen.

#### SUMMARY

Experimental conditions have been established for the elicitation of an allogeneic effect on the adoptive transfer secondary anti-2,4-dinitrophenyl (DNP) antibody response in mice. Thus, spleen cells from DNP-keyhole limpet hemocyanin (KLH)-primed mice manifest good secondary anti-DNP

<sup>3</sup> Katz, D. H., W. E. Paul, and B. Benacerraf. 1972. Carrier function in antihapten antibody responses. VI. Establishment of experimental conditions for either inhibitory or enhancing influences of carrier-specific cells on antibody production. Manuscript submitted.

responses to a challenge with DNP-KLH, but not with DNP-bovine gamma globulin (BGG), after adoptive transfer to irradiated syngeneic recipients. However, a good adoptive transfer secondary anti-DNP response of such cells can be elicited with DNP-BGG when a second transfer of allogeneic lymphoid cells, in appropriate numbers, is carried out 24 hr before secondary challenge. The advantage to this system is that the DNP-primed cell population as well as the population of allogeneic lymphoid cells are accessible to experimental manipulation such that the T lymphocytes of one or the other can be removed.

Utilizing this model, we have established that the allogeneic effect on antibody production can operate on a population of primed B lymphocytes which have been depleted of their isologous T lymphocytes by *in vitro* incubation with anti- $\theta$  serum plus complement. The potential cellular interactions involved in the mechanism of this phenomenon are considered and discussed in detail.

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