CELL INTERACTIONS IN THE IMMUNE RESPONSE IN VITRO

IV. COMPARISON OF THE EFFECTS OF ANTIGEN-SPECIFIC AND ALLOGENEIC THYMUS-DERIVED CELL FACTORS*

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Various theories have been proposed to explain the mechanism of collaboration between thymus-derived (T) and nonthymus-derived (B) lymphocytes in the induction of antibody responses. Among these is the possibility that T cells activated by antigen secrete a soluble factor capable of facilitating the induction of antibody production by B cells. In a system involving allogeneic combinations of lymphoid cells, Dutton et al. (1) in vitro and Katz et al. (2) and Kreth and Williamson (3) in vivo have obtained evidence for the release by T cells of a nonspecific factor stimulating B cell responsiveness. In these experiments T cells were activated by histocompatibility antigens, whereas the response of B cells to other antigens, such as heterologous erythrocytes or hapten-protein conjugates, was assayed. The recent demonstration by Schimpl and Wecker (4) of the production by alloantigenically stimulated T cells of a factor which could partially replace T cell function in the sheep red cell response has provided more direct evidence for the existence of a nonspecific component in T cell helper activity.

In contrast, Feldmann and Basten (5, 6) have found that T cells activated by a particular antigen elaborated a factor capable of triggering syngeneic B cells, but only

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1Abbreviations used in this paper: AF, allogeneic factor; AFC, antibody-forming cells; ATC, activated thymus cells; ATXBM, adult thymectomized, lethally irradiated, and bone marrow-protected; B lymphocytes, nonthymus-derived lymphocytes; CG POL, chicken globulin-coated polymeric flagellin; DNP FyG, dinitrophenylated chicken globulin; DNP Flg, dinitrophenylated flagella of Salmonella adelaide; DNP KLH, dinitrophenylated keyhole limpet hemocyanin; DRC, donkey red cells; KLH, keyhole limpet hemocyanin; POL, polymeric flagellin; SF, antigen-specific factor; SRC, sheep red cells; T lymphocytes, thymus-derived lymphocytes.
B cells which are reactive to determinants present on or linked to that particular antigen molecule. Thus this factor, produced by specific T cells, had effects only on specific B cells. Because the specificity of collaborative responses in vivo (7, 8) is known to be dictated by both classes of lymphocytes, it was hard to reconcile a major physiological role for a nonspecific mediator such as produced in detectable amounts in an allogeneic reaction with the known specificity of primary and secondary immune responses and immunological tolerance. Furthermore, a specific mechanism for T-B cell interaction seems more consistent with the requirement for linked recognition of T and B cell determinants (9). Although the properties of this antigen-specific factor were not characterized in detail, it did appear to have a relatively high molecular weight since it passed through a nuclo pore membrane of 0.1 μ pore size, but not through a dialysis membrane (6).

The purpose of the present work was to determine whether the stimulating factor released in an allogeneic reaction was the same or different from the cooperative factor found in syngeneic systems. The findings obtained demonstrate the existence of two distinct subcellular factors, differing both physicochemically and functionally, which augment antibody responses in vitro. The data suggested that an "antigen-specific" factor appears to be an initiating or immunizing agent, whereas the factor produced in allogeneic reactions apparently acted at a later stage in the immune response, probably augmenting the response by increasing the proliferation of already immunized B cells.

Materials and Methods

Animals.—Mice of the highly inbred CBA/H/Wehi and C57BL strains and noninbred congenitally athymic "nude" nu/nu mice were used. The origin and breeding of the nude mice, generously provided by Dr. M. C. Holmes, has been described in detail elsewhere (10).

Antigens.—Suspensions of sheep red cells (SRC) and donkey red cells (DRC), dinitrophenylated flagella of Salmonella adelaide (DNP Fla) with an average of 1.3 DNP groups per mole of monomeric flagellin, dinitrophenylated chicken globulin (DNP FγG), and dinitrophenylated keyhole limpet hemocyanin (DNP KLH) were prepared and used as outlined in a previous paper (11). Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem, Sydney, Australia. Chicken globulin-coated polymeric flagellin (CG POL) was prepared by incubating chicken anti-POL serum with polymeric flagellin (POL) at 37°C. A precipitate formed, which was washed, as described by Schrader and Feldmann.2

Tissue Culture.—Mouse spleen cells were cultured in a modified Marbrook-Diener system as described elsewhere (12). For experiments in which T cells and B cells were physically separated from each other, culture chambers with two distinct compartments were constructed according to the method described by Feldmann and Basten (6). T cells were placed in the upper compartment and a lymphoid suspension containing B cells and macrophages in the lower chamber. The two chambers were separated by a cell impermeable barrier, composed either of a nuclo pore membrane 0.2 μ pore size (General Electric Company, Schenectady, N.Y.) or a dialysis membrane (Union Carbide Corp., New York). Tests for leakage of cells through the nuclo pore membrane have been described in detail in a previous paper (6).

Preparation of Activated Thymus Cells (ATC).—ATC were prepared according to the method of Miller and Mitchell (13) by injecting thymus cells and antigen into heavily irradiated

2 Schrader, J. W., and M. Feldmann. 1972. Manuscript in preparation.
(800 rads) syngeneic recipients. Details of cell and antigen dosage and of irradiation were described in a previous paper (14).

**Antibody-Forming Cell Assays.**—The technique of Cunningham and Szenberg was used (15). To detect DNP-specific antibody-forming cells (AFC), SRC were coated with DNP conjugated to rabbit anti-SRC Fab fragments as described elsewhere (16). AFC to fowl gamma globulin were detected using the technique of Miller and Warner (17). Only direct IgM responses are shown in this paper. "Enhanced" plaques, indicating an IgG response, occurred, but as these were only 20-50% of the IgM response and varied in parallel with it, these results are not shown.

**Depletion of Phagocytes from Spleen.**—The method of Shortman et al. (18) was used. Basically spleen cells suspended in 50% mouse serum were passed through a column of large glass beads at 37°C. The effluent was extensively depleted of phagocytes (18).

**Cortisone Treatment.**—Female CBA mice, aged 35–40 days, were injected with 1.5 mg of cortisone acetate (The Upjohn Co., Kalamazoo, Mich.). This resulted in a 75–85% diminution in thymus cell numbers (19).

**Thymectomy.**—Adult thymectomized, lethally irradiated, and bone marrow-protected (ATXBM) mice were produced and used as described elsewhere (20).

### RESULTS

**Augmentation of the SRC Response of nu/nu Spleen Cells by Allogeneic T Cells.**—Reports of enhanced antibody production in histoincompatible situations both in vivo (21) and in vitro (14) prompted a comparison of the augmented responses in allogeneic situations with augmented antibody production in collaborative systems in vitro. Spleen cell populations from nude mice, which contain no T cells as judged by both functional (10) and alloantigenic (22) criteria, provided a source of B lymphocytes histoincompatible with CBA T cells. The collaborative capacity of specifically activated allogeneic T cells was assessed by measuring the SRC response of nu/nu spleen cultured with various numbers of ATC<sub>SRC</sub>. As controls, allogeneic cells activated to an unrelated antigen, KLH, were used. As shown in Fig. 1, CBA thymus cells activated to SRC or KLH both markedly enhanced antibody production to SRC, but the ATC<sub>SRC</sub> were more effective (*P* < 0.05). Even as few as 10<sup>5</sup> ATC<sub>SRC</sub> yielded responses greater than 10<sup>7</sup> ATC<sub>KLH</sub>.

**Specificity of Augmented Responsiveness in an Allogeneic Cell Mixture.**—Although ATC<sub>KLH</sub> were less effective than specific ATC<sub>SRC</sub> in augmenting the SRC response of nu/nu spleen cells, a highly significant number of AFC (up to 10,000 per culture) was still obtained with ATC<sub>KLH</sub> used at the optimal number. The specificity of augmentation of the response in allogeneic cell mixtures was investigated by measuring antibody production to two antigens, in the same cultures, to SRC as well as DRC. A typical experiment is illustrated in Fig. 2 that indicates that both T cell populations, ATC<sub>SRC</sub> and ATC<sub>KLH</sub>, enhanced the response to DRC equally, whereas ATC<sub>SRC</sub> exerted an appreciably greater effect on the SRC response than did ATC<sub>KLH</sub>. These experiments, with non-overlapping dose-response curves and the markedly different cell numbers at the threshold of enhancement, suggested the existence of two different com-
Fig. 1. Response of nu/nu spleen cells to SRC in the presence of CBA-activated thymus cells. T cells activated to SRC augmented the response to SRC much more than allogeneic T cells activated to an unrelated antigen. Each value is the arithmetic mean of four cultures ± standard error of mean. In the absence of T cells, the SRC response of nu/nu spleen was 410 ± 140 AFC/culture.

Fig. 2. Comparison of the effect of allogeneic CBA T cells on the responses of nu/nu spleen with SRC and DRC. •, Response to SRC in presence of ATC_{SRC}; ○, response to SRC in presence of ATC_{KLH}; □, response to DRC in presence of ATC_{KLH}; ■, response to DRC in presence of ATC_{SRC}.
ponents, "allogeneic" and antigen-specific components, in these collaborative antibody responses. It should be noted that in syngeneic systems thymus cells activated to irrelevant antigens do not give markedly increased responses (14).

**Allogeneic Augmentation of Thymus-Dependent and Thymus-Independent Antibody Responses.**—The nonantigen-specific augmentation of thymus-dependent antibody production to DRC and SRC by the addition of allogeneic cells raised the question whether histoincompatible T cells could also enhance the response to thymus-independent antigens. For this purpose, thymus-independent DNP conjugates of polymeric flagellin were used, which induce optimal responses in vitro in the absence of carrier-reactive cells (23) and T

| Cells cultured          | Antibody response (AFC/culture) | SRC | DRC | DNP | CG |
|------------------------|---------------------------------|-----|-----|-----|----|
| nu/nu spleen           |                                 | 110 | 40  | 240 | 155|
| " " 3 x 10^6 thymocytes|                                 | 690 | 160 | 453 | ---|
| " " 10^6 thymocytes    |                                 | 1090| 380 | 810 | 490|
| " " 10^6 cortisone-resistant thymocytes | | 1240| 390 | 620 | ---|
| " " 3 x 10^6 thymocytes|                                 | 1860| 670 | 960 | ---|
| " " 10^5 ATC, KLH      |                                 | 1240| 480 | 610 | ---|
| " " 10^7 ATC, KLH      |                                 | 940 | 640 | 1040| ---|
| " " 10^7 spleen cells  |                                 | 660 | 250 | 438 | 350|
| " " 10^7 spleen cells  |                                 | 795 | 490 | 890 | 510|

11 x 10^5 nude spleen cells were cultured in single-chamber flasks for 4 days with DNP FlA (0.1 μg/ml), DRC (3 x 10^6), and CG POL (1 μg/ml) or with SRC (3 x 10^6). T cells were from CBA mice allogeneic to the non inbred nu/nu mice. For simplicity, standard errors are not shown. These ranged from 12 to 24% of the means.

cells (20), as well as CG POL, which is also thymus independent (J. W. Schrader and M. Feldmann, unpublished observations). As thymus-dependent antigens, SRC and DRC were used. Nu/nu spleen cells were used as a source of B cells, while various sources of allogeneic T cells were obtained from CBA mice. Thymocytes, cortisone-treated thymocytes, ATC, and spleen cells all increased the in vitro response of nu/nu spleen to DNP FlA (Table I). The complete lack of antigen specificity of the allogeneic effect was confirmed by including POL coated with CG and DRC in the same cultures as DNP FlA, and SRC in parallel cultures. Antibody production to all these antigens was augmented by approximately the same degree by the presence of allogeneic T cells (Table I).

**Cell Causing Allogeneic Augmentation.**—The above experiments using thymocytes, spleen cells, cortisone-treated thymus, and activated T cells suggested that histoincompatible T cells were responsible for allogeneic augmentation of the response. To confirm this, a T-depleted population of CBA spleen cells from
ATXBM donors was cultured with nu/nu spleen cells. As shown in Table II the CBA ATXBM cells failed to significantly enhance the antibody responses.

**Allogeneic Augmentation across a Cell Impermeable Membrane.**—In a previous paper (6) specific collaboration between ATC and either primed or unprimed B cells was demonstrated across a cell impermeable membrane, if the membrane was of sufficient porosity (6). Thus collaboration occurred when a nuclepore membrane of pore size 0.1 μ or greater was interposed between the T and B cells, but not if a dialysis membrane was present. To test whether the allogeneic augmentation was also mediated by a subcellular factor, ATCKLH or ATCκκκ were mixed with either CBA spleen, C57BL spleen, or nu/nu spleen cells and placed in the upper compartment of double-chamber cultures. Spleen cells from nude mice were placed in the lower compartment together with SRC or a mixture of DRC, DNP POL, and CG POL. The two cell populations were

| Cells cultured | Anti-SRC response (AFC/culture ± se) |
|----------------|--------------------------------------|
| 11 × 10^6 nude spleen | 322 ± 110 |
| 11 × 10^6 ATXBM spleen | 280 ± 120 |
| 11 × 10^6 nude + 10^5 ATXBM | 360 ± 140 |
| 11 × 10^6 “ + 10^6 “ | 410 ± 140 |
| 11 × 10^6 “ + 3 × 10^6 “ | 405 ± 160 |
| 11 × 10^6 “ + 10^7 “ | 542 ± 125 |
| 11 × 10^6 “ + 10^6 ATC-KLH | 3640 ± 920 |

Each value represents the arithmetic mean of four cultures ± the standard error of the mean.

either separated by a nuclepore membrane of pore size 0.1 μ or by a dialysis membrane. The results in Table III indicate that pronounced stimulation of the antibody responses of nu/nu spleen to all the antigens present in the lower chamber occurred (3-10-fold) with both types of membrane if there was an allogeneic mixture in the upper chamber. In other words, the allogeneic effect is also mediated by a subcellular factor, but this factor is of smaller size than the antigen-specific factor described previously.

**Effect of the Allogeneic Reaction on the Response to DNP PyG or DNP KLH.**—Spleen cells from nude mice, although containing no detectable T cells, still give a measurable but feeble response to "thymus-dependent" antigens such as whole SRC and DRC (Table I, reference 10) although the detected response may be to the thymus-independent component of SRC (references 10 and 21; J. S. Haskill, personal communication; Byrd, M. Feldmann, and Palmer, unpublished data). In contrast, normal antibody production to DNP on a thymus-independent carrier, such as POL, can be demonstrated in the same culture system. Because unprimed cells from a T-containing spleen cell population,
such as normal CBA spleen, do not produce significant responses in vitro to nonpolymeric hapten-protein conjugates such as DNP F_3,G, DNP KLH, or DNP monomeric flagellin (11, 23), these antigenic systems (DNP F_3,G and DNP KLH) offered a way of determining whether the allogeneic factor augmented immune induction or merely augmented the proliferation of B cells which have already been immunized. Thus the effect of ATC_F_3,G or ATC_KLH on the response of cells from nude spleen to various DNP conjugates was ascer-

**TABLE III**

| Culture chamber | Antibody response (AFC/culture) |
|-----------------|---------------------------------|
|                 | SRC | DRC | DNP | CG |
| Nucleopore membrane |     |      |     |    |
| ATC_KLH + CBA spleen | Nude spleen | 310 | 50 | 490 | 400 |
| " + C57BL spleen | " " | 1910 | 250 | 2840 | ---* |
| " + nude spleen | " " | 3760 | 355 | 4910 | 1260 |
| ATC_SRC + CBA spleen | " " | 670 | 40 | 510 | --- |
| " + C57BL spleen | " " | 3420 | 190 | 1640 | --- |
| " + nude spleen | " " | 2410 | 390 | 2600 | --- |
| Dialysis membrane |     |      |     |    |
| ATC_KLH + CBA spleen | Nude spleen | 175 | 25 | 400 | 310 |
| " + C57BL spleen | " " | 2740 | 290 | 1935 | 1820 |
| " + nude spleen | " " | 3190 | 195 | 3500 | 1600 |
| ATC_SRC + CBA spleen | " " | 340 | 35 | 620 | --- |
| " + C57BL spleen | " " | 3910 | 450 | 3580 | --- |
| " + nude spleen | " " | 4100 | 490 | 2670 | --- |
| Nil | " " | 360 | 80 | 510 | 440 |

* Each value represents the arithmetic mean of three to six cultures of 30 X 10⁶ nude spleen cells which were cultured together with either SRC or DRC, DNP POL, and CG POL for 4 days. 5 X 10⁶ CBA ATC were used together with 2 X 10⁶ spleen cells in the upper compartment. Similar results were obtained in two other experiments.

* Not performed.

Tained (Table IV). There was no anti-DNP response of nude spleen cells cultured in the absence of other cells to DNP KLH or DNP F_3,G in vitro, in contrast to the results obtained with DNP Fla. The presence of either allogeneic ATC_KLH or ATC_F_3,G augmented the response to DNP Fla, whereas only specifically activated thymus cells augmented the anti-DNP response to thymus-dependent DNP conjugates; i.e., ATC_KLH augmented the anti-DNP response to DNP KLH, but not to DNP F_3,G, despite the equal allogeneic augmentation of the response to DRC (Table IV). This experiment suggested that the allogeneic factor alone could not cause otherwise unresponsive B cells to respond to antigen, but augmented antibody responses initiated in other ways, e.g., thymus-independent DNP Fla or specific cooperation of ATC_KLH with DNP KLH.
Because the antigen-specific factor does not penetrate dialysis membranes, it is possible to compare the actions of the allogeneic factor with the combined effect of both the allogeneic factor and the antigen-specific factor. Experiments were thus performed to compare the response of nu/nu spleen cells with the thymus-dependent antigens, DNP KLH and DNP FγG, separated from activated thymus cells by either a dialysis or a nuclepore membrane. The response of nu/nu spleen cells to these antigens, in the presence of the allogeneic factor only (dialysis membrane) or of both the allogeneic factor and the antigen-specific factor from ATC (nuclepore membrane), was ascertained. In the absence of an allogeneic reaction, there was no response of nu/nu spleen cells to DNP FγG or DNP KLH, but there was a response to DNP Fla and a minimal response to DRC (Table V). In the presence of the allogeneic factor only in cultures with ATC_{KLH} and nude spleen in the upper compartment separated from the lower chamber by a dialysis membrane, the responses to DNP Fla and DRC were both significantly augmented, unlike those to DNP FγG or DNP KLH. In the presence of both allogeneic and antigen-specific factors, that is in cultures divided by a nuclepore membrane, the response to DRC and DNP Fla was augmented, as was the response to DNP KLH (but not DNP FγG) if ATC_{KLH} were used. These experiments suggested that the allogeneic factor alone was not sufficient to enable nude spleen to respond to DNP FγG or DNP KLH, but in combination with the antigen-specific factor good anti-DNP responses occurred.

**Allogeneic Augmentation of the Response of Purified Lymphocytes.**—Experiments reported in the accompanying article (24) have demonstrated that the

| Cells cultured | Antigen | Antibody response (AFC/culture ± SE) |
|----------------|---------|-------------------------------------|
| nu/nu spleen   | DNP FγG | 20 ± 20                             |
| "             | DNP KLH | 40 ± 30                             |
| "             | DNP Fla | 360 ± 110                           |
| "             | 10^6 DNP FγG | 560 ± 80                          |
| "             | 10^6 DNP KLH | 42 ± 27                           |
| "             | 10^6 DNP Fla | 1015 ± 165                         |
| "             | 10^6 DNP FγG | 51 ± 30                            |
| "             | 10^6 DNP KLH | 716 ± 165                          |
| "             | 10^6 DNP Fla | 1290 ± 210                         |

15 X 10^6 nu/nu spleen cells were cultured in single-chamber flasks with 3 X 10^6 DRC and either DNP FγG (1 µg/ml), DNP KLH (1 µg/ml), or DNP Fla (0.1 µg/ml). Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Analogous results were obtained in three other experiments.
### TABLE V

**Effect of Allogeneic Factors on Response to DNP-Protein Conjugates**

| Culture chambers | Antigen Membrane | Antibody response (AFC/culture) |
|------------------|------------------|-------------------------------|
|                  | Upper            | Lower            | DNP  | DRC  | DNP   | DRC  |
| ATC<sub>KLH</sub> nu/nu spleen DNP KLH Dialysis | 20    | 120   |
| " " " "        | " " " "        | DNP F<sub>γ</sub>G " " | 10    | 160   |
| " " " "        | " " " "        | DNP Fla " "     | 510   | 145   |
| ATC<sub>KLH</sub> + nude nu/nu spleen DNP KLH Dialysis | 100   | 890   |
| " " + " + " + " | " " + " + " + " | DNP F<sub>γ</sub>G " " | 80    | 910   |
| " " + " + " + " | " " + " + " + " | DNP Fla " "     | 1640  | 1240  |
| ATC<sub>FG</sub> + nude nu/nu spleen DNP KLH Nucleopore | 890   | 1640  |
| " " + " + " + " | " " + " + " + " | DNP F<sub>γ</sub>G " " | 0     | 890   |
| " " + " + " + " | " " + " + " + " | DNP Fla " "     | 2050  | 1080  |
| ATC<sub>FG</sub> + nude nu/nu spleen DNP KLH Nucleopore | 60    | 990   |
| " " + " + " + " | " " + " + " + " | DNP F<sub>γ</sub>G " " | 950   | 1230  |
| " " + " + " + " | " " + " + " + " | DNP Fla " "     | 1850  | 1180  |

30 × 10<sup>6</sup> nu/nu spleen cells were cultured for 4 days in the lower chamber together with 3 × 10<sup>5</sup> DRC and 1 μg/ml DNP KLH or DNP F<sub>γ</sub>G in 0.1 μg/ml DNP Fla. 2 × 10<sup>6</sup> ATC and 10<sup>6</sup> nude spleen were used in the upper compartment. For clarity only the arithmetic means are shown of six to nine cultures derived from three experiments.

### TABLE VI

**Effect of Allogeneic Reaction on Response of Lymphocytes from nu/nu Spleen**

| Cells cultured | Antigen | Antibody response (AFC/culture ± SE) |
|----------------|---------|-------------------------------------|
|                 | B       | T                                   | DNP | DRC  | DNP  | DRC  |
| nu/nu LC        | " "    | DNP Fla                             | 308 ± 110 | 0   |
| " " " "        | " "    | DNP KLH                             | 0   | 0    |
| " " " "        | " "    | ATC<sub>KLH</sub> DNP F<sub>γ</sub>G | 805 ± 210 | 40 ± 10 |
| " " " "        | " "    | DNP KLH                             | 25 ± 20 | 60 ± 25 |
| " " " "        | " "    | ATC<sub>FG</sub> DNP F<sub>γ</sub>G | 920 ± 160 | 80 ± 40 |
| " " " "        | " "    | DNP KLH                             | 35 ± 25 | 70 ± 30 |
| nu/nu spleen    | " "    | DNP Fla                             | 420 ± 110 | 80 ± 40 |
| " " " "        | " "    | DNP KLH                             | 20 ± 20 | 110 ± 30 |
| " " " "        | " "    | ATC<sub>KLH</sub> DNP F<sub>γ</sub>G | 1210 ± 115 | 860 ± 110 |
| " " " "        | " "    | DNP KLH                             | 840 ± 180 | 920 ± 90 |
| " " " "        | " "    | ATC<sub>FG</sub> DNP F<sub>γ</sub>G | 1380 ± 155 | 1260 ± 50 |
| " " " "        | " "    | DNP KLH                             | 35 ± 10 | 980 ± 40 |

13 × 10<sup>6</sup> nude spleen or lymphocytes (LC) from nu/nu spleen were cultured with or without 10<sup>6</sup> ATC and 3 × 10<sup>5</sup> DRC for 4 days in single-chamber flasks. Number of CBA ATC used was always 10<sup>6</sup>. The concentrations of DNP KLH and DNP F<sub>γ</sub>G were 1 μg/ml and of DNP Fla 0.1 μg/ml. Each value represents the arithmetic mean of four to eight cultures pooled from two experiments.
specific factor produced by activated thymus cells in the presence of antigen only induces antibody responses if macrophages are present in the lower compartment. This finding suggested that the antigen-specific factor acted through the agency of macrophages. It was thus of interest to determine whether the allogeneic factor acted directly on B lymphocytes or on macrophages. The capacity of allogeneic (CBA)-activated T cells to augment the response to DNP KLH and DNP Fla of lymphocytes obtained from nude spleen by the method of Shortman et al. (18) was measured (Table VI). Enhancement of the response to macrophage-independent antigens was found, such as DNP Fla (23), but not to macrophage-dependent antigens such as DNP KLH (25) or red cells. Thus the allogeneic factor, unlike the antigen-specific factor, acted directly on B lymphocytes.

DISCUSSION

Evidence has recently been accumulating indicating that T-B cell interaction required for the induction of some antibody responses is an active metabolic process (14, 26) mediated by subcellular factor(s) released from antigen-stimulated T cells (5, 6). The precise nature of the factor(s) involved is unknown. By using a syngeneic system in vitro we have demonstrated the existence of an antigen-specific mediator of relatively high molecular weight (5, 6). When, however, histoincompatible cells were combined in vitro or in vivo, several groups of workers found direct (4) or indirect (1-3) evidence for the elaboration by T cells of a nonantigen-specific stimulating factor. The present in vitro experiments reconcile these apparent discrepancies, by demonstrating the existence of both a nonspecific and an antigen-specific factor possessing distinctive features and different modes of action.

Allogeneic stimulation of B cells was achieved by mixing T cells of various kinds from CBA (H-2k) donors with spleen cells from unprimed nude mice. These animals, which lack detectable T cells, are not inbred and can be assumed to be histoincompatible with H-2k (10). An excellent augmentation of the response was achieved when CBA thymocytes (which in our hands are inactive in a syngeneic system [10]), cortisone-resistant thymocytes, activated T cells, or spleen cells were employed (Table I). In contrast, the mixture of B cells from CBA ATXBM spleens and nude spleen cells was ineffective (Table II), indicating that allogeneic augmentation requires the presence of T cells. A similar conclusion was reached by Schimpi and Wecker (4) using anti-θ serum and complement. The specificity of the allogeneic effect was investigated in two different ways: in the first, CBA T cells were cultured with B cells carrying different H-2 antigens on their surface, e.g., spleen cells from C57BL or nude mice. The immune responsiveness of nude B cells was enhanced to a similar degree by either allogeneic reaction (Table III). Thus an allogeneic interaction not antigenically related to the antibody-producing system increases the response of nu/nu spleen. Secondly, the antigenic specificity of the action of the
allogeneic augmentation was examined by measuring antibody production of an allogeneic mixture to several different immunogens, including heterologous erythrocytes (SRC and DRC), DNP Fla, and CG POL (Figs. 1 and 2; Table I). In contrast to results obtained in syngeneic collaborative systems (11), the responses to all these antigens were enhanced to a comparable degree if T cells were not activated to one of the test antigens. The lack of specificity of the augmentation effect was well illustrated by the marked augmentation observed in numbers of AFC to DNP when cultures were stimulated with the DNP haptenic determinants coupled to a thymus-independent carrier such as flagella (20).

The capacity of histoincompatible T cells to augment the response of B cells across a cell impermeable barrier in a double-chamber culture system (Table III) provided direct evidence for mediation of the allogeneic effect by a soluble factor. The question therefore arose whether this allogeneic factor (AF) and the antigen-specific factor (SF) responsible for collaboration in a syngeneic system were the same or were different. Several experiments suggested that the two factors were discrete entities. First, AF failed to display any degree of antigenic specificity either in its induction phase or the effector phase (Table III), and, unlike the SF, it passed through dialysis membranes. Furthermore, specifically activated thymus cells augmented the response of allogeneic cells to a particular antigen to a greater degree than nonactivated cells or cells activated by another (noncross-reacting) antigen. An additive effect was observed, which implied the existence of two factors rather than one (Fig. 1). It was, however, conceivable for specifically activated cells by an antigen to release more of the same factor than cells activated to other antigens. This possibility was excluded by a series of allogeneic dose-response experiments (Figs. 1 and 2) using two antigens in the same culture. These findings, although favoring the elaboration of two factors rather than one in the collaborative system under study, do not precisely define their site of action in the generation of AFC.

The specificity of SF for both T and B cells is comparable with the known specificity of collaborating systems in vivo as found by Miller and Sprent (26) and Mitchison (27) and thus SF is probably involved in the induction phase of T-B cell interaction. The allogeneic factor, on the other hand, would appear to lack the necessary specificity to do this. Its capacity to augment thymus-independent antibody responses such as DNP Fla, CG POL, POL of strain 871 (unpublished data), as well as thymus-dependent antibody responses is quite unlike the lack of action or sometimes suppression of T cells in thymus-independent antibody responses (11) and implied that the AF acted at a later stage in the generation of AFC, possibly as an augmentor of the proliferation of B cells which have already been triggered. Further experiments were designed to determine whether AF could enhance the in vitro response to antigens which cannot elicit in vitro responses in the absence of carrier-primed or activated T cells. It was found (Table IV) that the AF could not replace these specific T cells. Further evidence of the inability of the allogeneic factor
alone to permit the immunization of B cells was found by comparing the responses of nude spleen with DNP FyG or DNP KLH occurring in double-chamber flasks with either dialysis or nuclepore membranes. In the presence of both factors (nuclepore membrane) responses to DRC, DNP Fla, and DNP FyG or DNP KLH were augmented. In the presence of the allogeneic factor only (dialysis membrane) responses to both DRC and DNP Fla were augmented, but not those to DNP FyG or DNP KLH. The enhanced response to DRC in the same flasks which did not respond to DNP FyG or DNP KLH was taken as strong evidence that the allogeneic factor in the presence of antigen is not sufficient to immunize normal B cells (Table V). In other words, AF did not initiate antibody production in an unresponsive cell population. Presumably AF acts at a later stage of the response, when antigen-reactive cells had already been triggered by AF. Support for this suggestion stems from the data of Schimpl and Wecker (4), which show that AF exerts its optimal effect on the SRC response some days after initiation of the response. This is in contrast to the action of T cells in a syngeneic SRC cooperative response system, which is completed within 48 hr (14). The importance of AF in the induction phase of cell collaboration in vivo thus remains to be determined, although its capacity to augment already established responses seems apparent. It is interesting to speculate whether the AF may not have a more profound effect in situations such as homograft rejection or autoimmune disorders, including allogeneic disease (28).

These results would appear to be at variance with the induction of anti-DNP responses to nonimmunogenic DNP compounds in guinea pigs undergoing an allogeneic response (29). However it is possible that the cell proliferation which occurs in graft-versus-host responses may also give rise to specific helper cells.

The over-all conclusion from the present study is that activated T cells produce two distinct components, which both can have the same net effect of augmenting antibody formation. One of these components is of large molecular weight which will be identified in the next two papers of this series (24) as a complex of T cell monomeric IgM and antigen. The other is of lower molecular weight, is not antigen specific, is produced in large quantities by T cells stimulated by histoincompatible cells, but is also produced in response to other antigens (references 6 and 24; P. Adams and K. Shortman, personal communication). The former factor only acts early in the response, initiating antibody production through the agency of macrophages, whereas the nonantigen-specific factor works optimally later in the response.

SUMMARY

The role of soluble factors in cell collaboration was investigated by means of a tissue culture system in which populations of T and B cells were either incubated together or separated from each other by cell impermeable membranes.

3 Feldmann, M., R. E. Cone, and J. J. Marchalonis. 1972. Cell interactions in the immune response in vitro. VI. Mediation by monomeric T cell surface IgM. Manuscript in preparation.
Histoincompatible T cells were found to augment antibody responses to both thymus-dependent and thymus-independent antigens, whether they were in contact with B cells or not. The properties of the factor released by the T cells in the allogeneic mixture were compared with those of the previously reported antigen-specific mediator found in syngeneic collaborative antibody responses. Unlike the latter, the factor made in allogeneic responses failed to display any degree of antigen specificity either in its induction or in its action, enhancing responses to all the antigens present in the cultures to a similar degree. It was of lower molecular weight than the antigen-specific factor, because it could pass through dialysis membranes as well as nucelopore membranes, whereas the antigen-specific factor could only penetrate nucelopore membranes. Furthermore, the factor made in allogeneic reactions had a different site of action. It acted directly on B lymphocytes, whereas the antigen-specific component acts through macrophages. Although antigen in the presence of the allogeneic factor did not initiate antibody production, it augmented responses once they had been induced by a matrix of antigenic determinants, either mediated by the antigen-specific factor or directly by a thymus-independent antigen. It was therefore considered to act at a later stage of the antibody response, probably as a nonspecific stimulator of immune B cell proliferation. Observations that the effect on the allogeneic factor are more pronounced 2 days after the beginning of the response are in keeping with this interpretation.

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