MECHANISM OF ACTIVATION OF THE BONE MARROW-DERIVED LYMPHOCYTE

III. A DISTINCTION BETWEEN A MACROPHAGE-PRODUCED TRIGGERING SIGNAL AND THE AMPLIFYING EFFECT ON TRIGGERED B LYMPHOCYTES OF ALLOGENEIC INTERACTIONS*

By John W. Schrader

(From the Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Victoria, Australia)

(Received for publication 26 June 1973)

In the immune response to many antigens, it appears that bone marrow-derived (B) lymphocytes can only develop into antibody-forming cells (AFC) in the presence of thymus-derived (T) lymphocytes (1). One suggested mechanism of this T-cell "help" is a focusing of antigen onto the B-cell surface (2). Recent in vitro studies have been interpreted as indicating that the critical factor in B-cell activation by both thymus-dependent and thymus-independent antigens is presentation of antigenic determinants in a multivalent configuration of suitable epitope density (3, 4). In the case of thymus-dependent antigens the role of the T cell is postulated to be the creation of the correct matrix of antigenic determinants on the macrophage surface (3). On the other hand, there have been proposals that B-cell activation may require two separate "signals" (5), "signal one" being union of antigen with the B-cell immunoglobulin receptor, and "signal two" being either a T-cell product (6-11) or a factor released by the macrophage (12-14).

Recently I have described an in vitro situation in which B cells in the absence of T cells were able to be triggered by an antigen, freshly deaggregated fowl gamma globulin (FyG), which clearly interacted with the B cells while in a nonmultivalent form (13, 15). Such activation of the B cell in the absence of T cells required the presence of a second substance that for convenience can be termed a signal-two source. Flagellin from Saltnonella strains, both in its polymerized and monomeric forms, and bacterial lipopolysaccharide (LPS) all possessed signal-two activity in this system (16). This system that used spleen cells from congenitally athymic (nu/nu) mice (17) as a source of B cells seemed suitable for an assay of putative physiological sources of signal two,

* This is publication no. 1898 from The Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Victoria, Australia.

1 Abbreviations used in this paper: AFC, antibody-forming cells; CFA, complete Freund's adjuvant; DRC, donkey erythrocytes; FCS, fetal calf serum; FyG, fowl gamma globulin; FLA, flagella; LPS, bacterial lipopolysaccharide; 2-ME, 2-mercaptoethanol; PC, peritoneal cells; PEC, peritoneal exudate cells; PPB, proteose peptone broth; S/N, supernatants; SRC, sheep erythrocytes.
as in the absence of the substances mentioned above there is absolutely no response to F\textsubscript{y}G (13).

The aim of the present study was to ask whether activated macrophages or activated T cells functioned as sources of signal two. Supernatants of mixed lymphocyte reactions have been claimed to replace T-cell function in a primary in vitro response to heterologous erythrocytes (6, 7). However with this type of antigen T cell-deficient systems are not totally unresponsive (6, 7, 18), and thus true restoration of T-cell function is difficult to distinguish from an amplification of this “thymus-independent” residual response. Therefore the present study uses an allogeneic interaction as a source of nonspecific T cell-dependent factors and compares the effects using (a) F\textsubscript{y}G as the antigen where there is no residual response, and (b) heterologous erythrocytes where a small response in the absence of added factors is the rule.

Materials and Methods

Animals.—Congenitally athymic “nude” (nu/nu) mice from a closed but not inbred colony maintained by Dr. Margaret Holmes (19) were used between 6-8 wk of age. CBA/H/WEHI mice were used at 6-10 wk of age.

Tissue Culture.—The method of Marbrook as described elsewhere was used (4). Briefly, 15 X 10\textsuperscript{6} viable nucleated spleen cells plus antigens were cultured in 1 ml of tissue culture fluid, a dialysis membrane separating the cells from a reservoir of 50 ml of medium. The medium used was Eagle’s minimal essential medium with nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y.) to which was added 5% fetal calf serum (FCS) Commonwealth Serum Laboratories, Parkville, Melbourne, Australia). In some experiments, the medium was supplemented by 10\textsuperscript{-5} M 2-\beta-mercaptoethanol (2-ME). Cells were cultured for 3 or 4 days.

Antigens.—Fowl gamma globulin (F\textsubscript{y}G) and polymerized flagellin (POL) or flagella (FLA) were prepared as described elsewhere (13). Lipopolysaccharide (LPS) prepared from Salmonella typhimurium was obtained from Difco Laboratories, Inc. (Detroit, Mich.). Sheep erythrocytes (SRC) were obtained from two sheep maintained at the Hall Institute. Blood was stored in Alsever’s solution at 4°C for 1-2 wk before use. SRC were washed three times in normal saline before addition to cultures. Donkey erythrocytes (DRC) were obtained from an animal maintained by Commonwealth Serum Laboratories and were stored at 4°C for 4 wk.

Assays for Antibody-Forming Cells (AFC).—Anti-F\textsubscript{y}G AFC were detected using a modified hemolytic plaque technique (13). Anti-SRC and anti-DRC AFC were detected using the appropriate erythrocytes in a modified hemolytic plaque assay (19). Results are expressed as the arithmetic means of groups of four cultures plus or minus the standard error of the mean.

Production of Peritoneal Exudate Cells (PEC).—Mice, either CBA or nu/nu, were injected with 1 ml of sterile proteose peptone broth (PPB) (Difco Laboratories) and 4-5 days later, peritoneal exudate cells were harvested in Dulbecco’s solution. In certain experiments nu/nu mice were intraperitoneally injected with 0.1 ml of complete Freund’s adjuvant (CFA) (Difco Laboratories).

Preparation of Peritoneal Cell (PC) Supernatants (S/N).—Normal CBA or nu/nu mice were used as the source of peritoneal cells (PC) obtained by repeated washings of the peritoneal cavity with Dulbecco’s solution. PC were washed once through an FCS underlayer. From 2 to 10 X 10\textsuperscript{5} PC were cultured for 16-24 h in small 35 x 10 mm plastic Petri dishes (1008; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). In some cases FCS was omitted from the washing procedure and subsequent culture.
Mitomycin C Treatment of CBA Spleen Cells.—30 × 10^6 CBA spleen cells were incubated for 30 min at 37°C in medium containing 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.) after which the cells were washed through an FCS underlayer (20).

Trypsin Treatment of Supernatants.—Crystalline trypsin (Grade A; Calbiochem San Diego, Calif.) was added to supernatants to give a final concentration of 100 μg/ml. After incubation for 2 h at 37°C, trypsin inhibitor (from Soya bean type 1-S; Sigma) was added to 100 μg/ml. Controls showed that trypsin plus inhibitor at the concentrations present in cultures given trypsin-treated supernatants in this experiment do not inhibit antibody responses.

RESULTS

The Effect of the Addition of nu/nu PEC on the Response of nu/nu Spleen to F'γG.—As a step in investigating the possible role of the activated macrophage in the triggering of the B cell by a thymus-dependent antigen, peritoneal exudate cells (PEC) were added to cultures of nu/nu spleen cells together with an optimal dose of F'γG. Since the PEC were obtained from nu/nu mice, which are devoid of functional T cells (18), no T cells were being thus added to the cultures. Similarly no T cell function could have been involved in activation of the PEC.

Table I demonstrates that the addition of PEC from nu/nu mice allowed

| TABLE I |

PEC from Nude Mice Allow a Response to FγG

| Added to cultures in addition to F'γG | Anti-F'γG AFC/culture |
|------------------------------------|----------------------|
| Exp. I                             |                      |
| —                                  | 0                    |
| POL 10 μg                          | 514 ± 66             |
| PEC 2.0 × 10^6                     | 362 ± 102            |
| " 0.25 × 10^6                     | 214 ± 42             |
| Exp. II                            |                      |
| —                                  | 0                    |
| POL 10 μg                          | 369 ± 50             |
| (a) PEC 2 × 10^6                   | 204 ± 23             |
| " 10^6                           | 138 ± 42             |
| " 10^5                           | 72 ± 15              |
| " 10^4                           | 18 ± 13              |
| (b) PEC 10^6                      | 165 ± 40             |
| " 10^5                           | 165 ± 18             |
| " 10^4                           | 24 ± 20              |

Spleen cells from nu/nu mice were cultured in the presence of 100 μg/ml F'γG, plus either POL or PEC, from nu/nu mice injected 4 days earlier with 1 ml of PPB. The PEC were incubated for 1½ h with the F'γG before adding the spleen cells. The medium contained 2-ME (10⁻⁵ M). 4 days later the cells were harvested and assayed for anti-F'γG AFC.

In one part, (a), of exp. II, nu/nu PEC were added in varying numbers to a mixture of the spleen cells and the antigen, while in the second part, (b), the PEC were incubated as in exp. I with the F'γG first.
nu/nu spleen cells to respond to F\gamma G. Clearly the factor allowing the B cells to be activated by F\gamma G was not the product of T cells and nor in this experimental design was it T cell dependent.

The Effect of Unstimulated Peritoneal Cells from Nude Mice.—When normal, untreated nude mice were used as a source of peritoneal cells, as Table II shows, the responses were negligible. Thus it seemed that the injection of PPB resulted in either migration of some new cell type into the peritoneal cavity or the activation of a cell type already present, thereby allowing the peritoneal cell population to act as a source of signal two for B-cell activation. That the relevant cell type or a precursor was present in the unstimulated PC population was shown by subsequent experiments demonstrating that such PC could be stimulated in vitro to release a substance with signal-two activity.

The Activity of Complete Freund’s Adjuvant in Stimulating the Macrophage.—

### Table II

| Added to cultures in addition to F\gamma G | Anti-F\gamma G AFC/culture |
|------------------------------------------|---------------------------|
| —                                        | 6 ± 6                     |
| PC 10^6                                  | 12 ± 11                   |
| 10^5                                     | 6 ± 5                     |
| 10^4                                     | 0                         |
| POL 10 µg                                | 214 ± 53                  |

Spleen cells from nu/nu mice were cultured with 100 µg/ml F\gamma G plus either peritoneal cells from unstimulated nu/nu mice or, as controls, POL 10 µg or nothing. Cells were harvested after 3 days. Similar results were obtained in experiments where the medium was supplemented with 2-ME (10^-5 M).

Many adjuvants have stimulatory effects on the macrophage (e.g. 21, 22), but a characteristic of T-deprived animals is that even with stimulation by complete Freund’s adjuvant (CFA), little antibody may be produced to T cell-dependent antigens. Therefore it was of interest to see whether CFA could activate peritoneal cells in nu/nu mice in the same manner as had been demonstrated for PPB. Table III shows that CFA in a typical adjuvant dose was a poor inducer in peritoneal cells of the capacity to permit nu/nu spleen cells to respond to F\gamma G. The minimal effects of peritoneal cells from normal animals seen in this experiment are of interest. These may reflect some natural activation of peritoneal cells by infection.

The Effect of Allogeneic PEC on the Response of nu/nu Spleen to F\gamma G.—The nu/nu mice used are not inbred and most cells in pools of nu/nu spleen cells will exhibit some histocompatibility products (mostly BALB/c type) that differ from those on CBA cells and some that are shared in common with CBA cells (18). Therefore CBA PEC were used to test whether some degree of histo-
MECHANISM OF ACTIVATION OF THE B LYMPHOCYTE

TABLE III
A Comparison of PPB and CFA as Activators of nu/nu PC

| Added in addition to FγG | Anti-FγG AFC/culture |
|-------------------------|----------------------|
|                         | 0                    |
| (a) 10^6 PEC (PPB stimulated) | 256 ± 35 |
| 10^5 “ “ “ | 87 ± 24 |
| (b) 10^6 PEC (CFA stimulated) | 90 ± 36 |
| 10^5 “ “ “ | 46 ± 26 |
| (c) 10^6 PC unstimulated | 54 ± 42 |

POL 10 μg | 381 ± 20 |

Spleen cells from nu/nu mice were cultured with FγG 100 μg/ml plus PEC as indicated from nu/nu mice injected 4 days previously with either 1 ml of PPB (a), or 0.1 ml of CFA (b), or peritoneal cells (PC) washed from normal nu/nu mice (c). Cells were harvested after 3 days.

incompatibility was a barrier in this system. CBA PEC were cultured for 3 days after which period the morphology of viable cells was predominantly that of active macrophages. Such cell populations were capable of allowing nu/nu spleen cells to respond to FγG (Table IV).

The Effect of Supernatants of Peritoneal Cells.—As a first step in an analysis of the role of the macrophage in B-cell triggering, the effect of supernatants from cultures of peritoneal cells on the response of nu/nu spleen cell cultures to FγG was investigated. Table V shows that peritoneal cells were releasing a factor into the culture medium that facilitated the activation of B cells by FγG.

Stimulation of PC by LPS.—Supernatants of cultures of PC that had contained 3 μg/ml of LPS were also active (Table V). Comparison of the effect on the response to FγG by cultures of nu/nu spleen, of 0.5 ml of supernatant from CBA PC cultures containing LPS (1.5 μg) with the effect of 3 μg of LPS added directly to cultures, shows that the supernatant-treated cultures responded twice as well as those receiving LPS alone. Likewise 0.5 ml of supernatant of cultures of CBA PC cultured with LPS (LPS-S/N-CBA) allowed a response to FγG, of about twice the magnitude of that developing in the presence of 0.5 ml of supernatants of CBA PC cultured in medium alone. Since the anti-FγG AFC response with 0.5 ml of LPS-S/N-CBA, at a mean of 672 AFC/culture, is of the order of the maximal seen with 100 μg of FγG in the presence of optimal doses of POL or LPS (10 μg/ml), and since the effect of higher doses of these latter substances is to produce a plateau-level response to FγG (13, 16), it was not possible to assume that a simple addition of the effects of the LPS and the CBA-PC-supernatant had resulted in this response. In fact the re-
JOHN W. SCHRADE

1471

TABLE IV

Allogeneic PEC Allow Triggering of nu/nu B Cells by FyG

| CBA PEC/culture | Anti-FyG AFC/culture |
|-----------------|----------------------|
|                 | 0                    |
| 10^6            | 288 ± 72             |

PEC from CBA mice were cultured for 3 days and added to cultures of nu/nu spleen cells together with 100 μg of FyG. Control cultures contained 100 μg of FγG alone. The medium contained 2-ME (10^{-5} M) and the spleen cells were harvested after 3 days.

TABLE V

The Signal-Two Effect of Supernatants of Cultures of Peritoneal Cells

| Cultures contain 100 μg FyG plus | Anti-FyG response AFC/culture |
|----------------------------------|------------------------------|
| POL 10 μg                        | 618 ± 180                    |
| 0.5 ml S/N-CBA                   | 369 ± 54                     |
| 0.05 ml S/N-CBA                  | 247 ± 78                     |
| (b) 0.5 ml S/N-CBA (trypsinized) | 90 ± 48                      |
| 3 μg LPS                         | 316 ± 60                     |
| (a) 0.5 ml LPS-S/N-CBA           | 672 ± 66                     |
| (a) 0.05 ml LPS-S/N-CBA          | 513 ± 84                     |
| (b) 0.5 ml LPS-S/N-CBA (trypsinized) | 306 ± 60             |
| (c) 3 μg LPS (trypsinized)       | 445 ± 74                     |
| (a) 0.5 ml LPS-S/N (nu/nu)       | 630 ± 48                     |
| (a) 0.05 ml LPS-S/N (nu/nu)      | 342 ± 132                    |

Spleen cells from nu/nu mice were cultured with FγG (100 μg/ml) plus supernatants of overnight cultures of 10 × 10^6 PC from either CBA (S/N-CBA) or nu/nu (S/N-nu/nu) mice in 2 ml of medium containing 5% FCS. To some CBA PC cultures and all (nu/nu) PC cultures were added LPS (3 μg/ml), and nu/nu spleen cell cultures given 0.5 and 0.05 ml (a) of supernatants of these PC cultures contained a maximum of 1.5 and 0.15 μg of LPS, respectively. Trypsin treatment was performed as described in the Materials and Methods section and treated supernatants were added as indicated (b). As a control LPS, 3 μg, was also trypsinized and added to cultures (c). Cultures were harvested after 3 days.

Responses of cultures to which 0.05 ml of LPS-S/N-CBA had been added showed a synergistic effect to be in operation. Here the amount of LPS added per culture was only 0.15 μg, an amount alone allowing a barely detectable response to 100 μg of FγG (16). However the anti-FγG response was still at a maximal level, which was twice that of cultures given 0.05 ml of S/N from PC cultured without LPS. PC from nu/nu mice also produced active supernatants,
showing that the production of the relevant factor was T-cell independent (Table V).

Treatment of PC Supernatants with Trypsin.—As a preliminary investigation of the chemical nature of the factor in PC supernatants with signal-two activity, the effect of treating PC supernatants with trypsin was investigated. Table V demonstrates that the activity of 0.5 ml of the supernatant of cultured CBA PC was reduced 75% by trypsin treatment. Similarly, the increase in activity of supernatants of PC cultured with LPS, over the activity of 3 μg of LPS alone, was abolished. Control cultures where LPS was treated with trypsin demonstrated that the signal-two activity of LPS is unaffected by proteolytic enzymes.

The Effect of Omitting FCS in Preparation of PC Supernatants.—Merely culturing PC seemed to be an adequate stimulus for the production of a factor allowing nu/nu spleen cells to respond to FγG. Apart from the simple chemicals constituting the medium, the possible stimuli were the plastic dish or the FCS. Therefore, the effect of omitting the FCS from the medium in which PC were cultured was investigated. Table VI shows that FCS appeared to be a stimulatory influence on the production by cultured PC of supernatants allowing nu/nu spleens to respond to FγG.

The Effect of Addition of Allogeneic Spleen Cells to the Response of nu/nu Spleen to FγG.—Allogeneic interactions have been shown to circumvent the requirement for the presence of specific T cells in in vivo secondary responses (23) and primary responses to one antigen with special properties, DNP-d-GL (DNP conjugate of a synthetic random copolymer of d-glutamic acid and d-lysine) (24). In vitro studies Schimpl and Wecker have shown that a factor released from allogeneically stimulated T cells can reconstitute the response of T cell-deficient spleen cell cultures to SRC (7). An allogeneic interaction between CBA spleen cells and spleen cells from the noninbred nu/nu mice (25) was used as the basis of the following experiments (Table VII) that were designed to examine the effect of T cell-dependent factors on antigen-induced

| TABLE VI |
| --- |
| The Effect of FCS in Stimulating PC |
| Supernatant from | Anti-FγG AFC/culture |
| nu/nu PEC + 5% FCS | 193 ± 54 |
| nu/nu PEC (no FCS) | 54 ± 33 |
| (a) | --- |

Normal nu/nu mice were the source of peritoneal cells that were cultured (2.10⁴) in small plastic Petri dishes for 20 h in either 2 ml of normal tissue culture medium with 5% FCS or 2 ml of medium without FCS. To each culture of nu/nu spleen cells was added FγG (100 μg) plus 0.25 ml of supernatants of the PC obtained with or without FCS being present. A control group of cultures contained FγG alone (a).
TABLE VII

The Requirement for the Presence of FLA in the Enhancing Effect of Allogeneic Spleen Cells on the Response of B Cells to F'yG

| FLA          | CBA spleen cells | Anti-F'yG AFC/culture |
|--------------|------------------|-----------------------|
| -            | -                | 0                     |
| +            | -                | 163 ± 60              |
| +            | 10⁴              | 198 ± 35              |
| +            | 10⁵              | 286 ± 114             |
| +            | 10⁶              | 913 ± 150             |
| -            | 10⁶              | 0                     |

Spleen cells from nu/nu mice were cultured with 100 μg of F'yG plus graded numbers of allogeneic CBA spleen cells from normal mice as indicated. Flagella (FLA) were present (10 μg/ml) as indicated. Cultures were harvested after 4 days.

triggering in this system. The addition of small numbers of CBA spleen cells to cultures of nu/nu spleen containing F'yG 100 μg/ml did not reverse the inability of nu/nu spleen cells to respond to F'yG. However a clear enhancing effect on the anti-F'yG response was seen when increasing numbers of CBA spleen cells were added to cultures containing, in addition to F'yG, a substance having signal-two activity, such as POL or FLA (13). Thus the conclusion seemed clear that the addition of CBA spleen cells had an effect on only B cells that had been already activated by F'yG plus signal two. Alone, an allogeneic interaction, despite its profound amplifying effect on the AFC response eventuating from F'yG-reactive B cells that had been already activated, had no influence as a second signal in the triggering event.

The Effect of Mitomycin C Treatment of the CBA Spleen Cells.—There is evidence (reviewed by Miller [1]) that unimmunized T cells must undergo division before being effective as helper cells. Therefore CBA cells were preincubated with a dose of mitomycin C shown to be sufficient to totally inhibit [3H]-thymidine uptake in cultures of spleen cells (20) in order to exclude the possibility that specific helper T cells had been activated in the presence of POL. Table VIII shows that mitomycin C-treated CBA spleen cells were just as effective as untreated CBA spleen, indicating that proliferation of the T cells was not required for the allogeneic effect.

The Synergistic Effect of Allogeneic Spleen Cells Plus PC Supernatants on the Response to F'yG of nu/nu Spleen.—To test the hypothesis that a factor in supernatants from cultured PC was providing a substance important in B-cell triggering, while allogeneic interactions were releasing factors amplifying the effects of B-cell triggering in terms of numbers of resultant AFC, the effect on the response to F'yG of adding such PC supernatants plus allogeneic spleen cells to cultures of nu/nu spleen was examined. Fig. 1 depicts the results of
MECHANISM OF ACTIVATION OF THE B LYMPHOCYTE

Fig. 1. Synergism between supernatants of PC and an allogeneic interaction in the anti-FyG response of nu/nu spleen. Groups of four cultures of nu/nu spleen were set up each with 100 μg/ml FyG plus additional factors as indicated. The dose of S/N was 0.5 ml of an overnight culture of PC. In one group this was treated with trypsin (as in Materials and Methods), before addition to the cultures. The allogeneic interaction was provided by 10⁶ mitomycin C-treated CBA spleen cells added to each culture.

such an experiment. Clearly in the presence of such supernatants, nu/nu cultures given allogeneic CBA spleen cells were able to respond to FyG. It was also evident that responses in the presence of CBA cells plus supernatant and FyG were higher than those of cultures with supernatant and FyG alone. Thus the same synergism was apparent between the allogeneic effect and macrophage supernatants, as had been found between the allogeneic effect and POL (Table VII). This particular experiment also showed again that trypsin diminished the stimulatory activity of macrophage supernatants.

The Effect of POL and Allogeneic-Recognition Factor on Responses of nu/nu Spleen to Heterologous Erythrocytes.—For the purposes of comparison with the data obtained with FyG, the effect of these two influences on the in vitro response of nu/nu mice to a second class of thymus-dependent antigens, heterologous erythrocytes (1), was examined. Table IX shows the results of experiments with DRC and SRC. In contrast to the FyG system, there is a definite response of the T cell-deficient nu/nu spleen cells to both SRC and DRC in the absence of the addition of any second substance. Addition of POL does
TABLE VIII

The Undiminished Activity of Mitomycin C-Treated Allogeneic Spleen Cells in Amplifying the Response of B Cells to FyG in the Presence of POL

| POL | CBA spleen cells (10⁶/culture) | Anti-FyG AFC/culture |
|-----|-------------------------------|---------------------|
| −   | −                             | 0                   |
| +   | −                             | 204 ± 42            |
| +   | Normal                        | 1,218 ± 198         |
| +   | Mitomycin C treated           | 1,470 ± 264         |
| −   | Normal                        | 0                   |
| −   | Mitomycin C treated           | 0                   |

Spleen cells from nu/nu mice were cultured with FyG 100 µg/ml. CBA spleen cells, either treated with mitomycin C as described above or left untreated, were added to cultures as indicated. POL (10 µg/ml) was present as indicated. Cultures were harvested after 4 days.

TABLE IX

Enhancing Effect of both Allogeneic Spleen Cells Alone and POL on the Response of nu/nu Spleen Cells to Heterologous Erythrocytes

| Added to cultures | Anti-DRC AFC/culture | Anti-SRC AFC/culture |
|-------------------|----------------------|----------------------|
| −                 | 190 ± 100            | 209 ± 88             |
| 10 µg POL         | 952 ± 343            | 1,958 ± 440          |
| 10⁶ CBA spleen cells | 1,402 ± 473       | 2,939 ± 924          |

Spleen cells from nude mice were cultured with 3.10⁶ DRC and 3.10⁶ SRC. To some groups were added 10 µg of POL, to others 10⁶ CBA spleen cells, treated with mitomycin C as described above.

produce in both systems a large increase in numbers of AFC. However in contrast to the situation with FyG, the addition of allogeneic CBA cells alone without any POL being present markedly raised the numbers of AFC produced.

DISCUSSION

Spleen cells from nu/nu mice cannot respond in tissue culture to the antigen FyG by AFC production unless a source of some substance aiding in the triggering event (a signal-two substance) is added (13, 15, 16). This study has shown that activated peritoneal cells, but not freshly harvested, unactivated peritoneal cells, allow cultured nu/nu spleen cells to respond to FyG. Since the PEC population that was active after preliminary culture (Table IV) consisted almost entirely of active macrophages, these are most likely to be the relevant cell type. Supernatants of cultured peritoneal cells were able to substitute for PEC and the activity of these supernatants was sensitive to trypsin, suggesting that the active factor is a protein. Because the peritoneal cell populations could be obtained from nu/nu mice, it can be concluded that the activation of macro-
phages to release such a factor does not require T cells, at least under the present experimental circumstances.

The stimulatory action of PPB on PC in vivo may well reflect some content of endotoxin, a well-established macrophage stimulant (26 and Table V). The minimal stimulatory action of PC in vivo of CFA is somewhat unexpected but quite consistent with its ineffectiveness in contrast to POL as an adjuvant for the anti-F\(\gamma\)G response of nu/nu mice in vivo (unpublished data). The increased release of signal-two material from cultured PC in the presence of FCS was of interest. This probably represents a true stimulation of macrophages, as other studies have shown that FCS raises levels of cathepsin D and acid phosphatase in cultured peritoneal cells (27).

Recently it has been proposed that the second signal for B-cell activation is provided by the interaction of the complement component C3b with the B-cell surface (14). It is possible that LPS and POL and even monomeric flagellin (16) are acting via C3 activation in this system. Likewise the supernatant factor could be a C3-splitting protease, although as yet there is no pertinent evidence. Conversely it is conceivable that the role of C3 activation in the immune response could be in the activation of the macrophage to secrete a signal-two substance.

The second part of this study showed that the addition of living allogeneic T cells to cultures of nu/nu spleen with F\(\gamma\)G could not by itself facilitate the generation of anti-F\(\gamma\)G AFC. In contrast if such a source of allogeneic factors (6, 7, 25) was added to cultures containing POL or macrophage supernatants in addition to F\(\gamma\)G, a greatly augmented production of anti-F\(\gamma\)G AFC was seen. These experiments therefore differentiate two forms of stimulatory activity in this in vitro system namely, (a) a second-signal factor produced by activated macrophages that allows an antigen-B-cell interaction to proceed to B-cell activation and (b) a T cell-dependent amplifying factor that is produced by allogeneic interaction, and which while not itself involved in B-cell triggering, does amplify the number of AFC produced (25, 27–29) perhaps by a mitogenic effect (30).

The present study confirms previous reports (6, 7) that allogeneic effects "restore" the responses of T cell-deficient cultures to heterologous erythrocytes. However, in contrast to the case with F\(\gamma\)G, nu/nu spleen cells challenged with heterologous erythrocytes alone do give a small but definite response (Table IX). Thus the restoration by allogeneic factors may be explained by an amplification of this basal thymus-independent response without any influence on B-cell activation.

Why are heterologous erythrocytes able to trigger a significant AFC response in nu/nu spleen cells without addition of any signal-two source? Of several possibilities that suggest themselves two seem especially worthy of exploration. Firstly, such responses may involve a different subpopulation of "memory" B cells with prior experience of cross-reacting antigens (31) and thus be
triggered according to different rules. Secondly, particulate antigens such as erythrocytes could during the process of phagocytosis activate the macrophage to release a signal-two substance. The small response to erythrocyte antigens in T cell-deficient mice (e.g. 18, 32) stands in contrast to the response to more conventional thymus-independent antigens. A probable reason is that the latter in general are bacterial products or artificial substances (33) that persist for prolonged periods in the body due to their nondegradability. It is suggested that these substances are able to provide signal two for B-cell activation, either by direct interaction with the B cell or by activating the macrophage, a function perhaps facilitated by their nondegradability.

The present study has shown that B-cell triggering, resulting at least in an IgM response to a thymus-dependent antigen, need not involve the T cell. It suggests that the activated T cell has a nonspecific amplifying influence on B-cell responses, but that this affects only those B cells that have been activated by a separate mechanism. The data are compatible with the hypothesis that the B cell is triggered at the macrophage surface where it encounters (a) antigen and (b) a short-ranged second-signal substance secreted by the macrophage. It is worth noting that the T cell may also be activated by antigen presented by the macrophage (reviewed in 34, 35) perhaps with the involvement of a soluble factor (36, 37).

How can demonstrations of carrier specificity be explained? Firstly there could be a requirement in vivo for the anatomical proximity of the B cell, macrophage, and T cell. Secondly the T cell may have an active role in actual B-cell triggering through a focusing of antigen onto the macrophage surface (3).

The above hypothesis predicts that antibody responses resulting from the activation of the B cell in the absence of T cells will be deficient, because of the lack of the proliferative influence of the activated T cell. Also relevant is the role of the T cell in determining antibody affinity and class (1). Thus the results of Unanue (38), where antigen presented to thymectomized mice on activated macrophages did not alter the serum antigen-binding capacity, do not necessarily contradict the present study since in his study a small transient IgM response may not have been detected.

SUMMARY

Peritoneal exudate cells from nu/nu mice stimulated with proteose peptone broth, but in general not from unstimulated mice, permitted cultures of spleen cells from congenitally athymic (nu/nu) mice to respond to the thymus-dependent antigen fowl gamma globulin (F\textgamma G). Supernatants of cultures of peritoneal cells were also effective, the activity being sensitive to trypsin. Since nu/nu mice were effective sources of the peritoneal cells it would not seem obligatory for the thymus-derived (T) cell to be involved in the triggering of the bone marrow-derived (B) cell by a thymus-dependent antigen F\textgamma G. It is proposed that the B cell is triggered at the macrophage surface where it en-
counters two signals (a) the antigen and (b) a protein secreted by the activated macrophage. In vivo the T cell may have a role in B-cell triggering, either in activating the macrophage or in aiding in presentation of antigen on the macrophage surface. Thymus-independent antigens are proposed to induce an IgM response because they are able to provide "signal two" either by direct interaction with the B cell or via irritation or activation of the macrophage. The stimulatory effect of T cells activated by an allogeneic interaction was used as a model of one influence of the T cell on the development of an antibody response. The presence in cultures of nu/nu spleen of an allogeneic interaction had no effect on the inability of these cells to respond to F\gamma G. However when a source of the postulated second signal such as the supernatant of a macrophage culture was present, an allogeneic interaction had a powerful amplifying effect on the anti-F\gamma G response. In contrast the response of nu/nu spleen cultures to heterologous erythrocytes was greatly enhanced by the presence of an allogeneic interaction. It is suggested that since there was a definite basal response to the heterologous erythrocytes added alone, the enhancement represented not an activation of more B cells but rather an amplification of this basal response. Thus the anti-F\gamma G response in cultures of nu/nu spleen differentiates between factors such as those released by activated macrophages that are involved in B-cell triggering and factors released by activated T cells that amplify the numbers of antibody-forming cells resulting from a B cell already triggered.

The author is in receipt of a National Health and Medical Research Council (Canberra Australia) Medical Postgraduate Scholarship.

This work was supported by the National Health and Medical Research Council, Canberra, Australia and National Institutes of Health grant AI-0-3958, and was in part pursuant to contract no. NIH-HCI-G-72-3889 with the National Cancer Institute, National Institutes of Health.

I thank Professor G. J. V. Nossal for stimulating discussions, Dr. Margaret Holmes for supplying the nude mice, and Mr. John Pye for supplying the POL.

Miss Jillian Jackson provided excellent technical assistance.

REFERENCES

1. Miller, J. F. A. P. 1972. Lymphocyte interactions in antibody responses. Int. Rev. Cytol. 33:77.
2. Mitchison, N. A. 1971. Cell cooperation in the immune response: the hypothesis of an antigen presentation mechanism. Immunopathology. 6:52.
3. Feldmann, M. 1972. Cell interaction in the immune response in vitro. V. Specific collaboration via complexes of antigen and thymus-derived cell immunoglobulin. J. Exp. Med. 136:737.
4. Feldmann, M., and A. Basten. 1971. The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. J. Exp. Med. 134:109.
5. Cohn, M. 1971. The take home lesson. Ann. N.Y. Acad. Sci. 190:529.
6. Dutton, R. W., R. Falkoff, J. A. Hirst, M. Hoffman, J. W. Kappler, J. R. Kettman, J. F. Lesley, and D. Vann. 1971. Is there evidence for a non-antigen specific
diffusible chemical mediator from the thymus-derived cell in the initiation of the immune response? In Progress in Immunology. B. Amos, editor. Academic Press, Inc., New York. 335.

7. Schimpl, A., and E. Wecker. 1972. Replacement of T cell function by a T cell product. Nat. New Biol. 237:15.

8. Gorczynski, R. M., R. G. Miller, and R. A. Phillips. 1972. Initiation of antibody production to sheep erythrocytes in vitro: replacement of the requirement for T-cells with a cell-free factor isolated from cultures of lymphoid cells. J. Immunol. 108:457.

9. Kreth, H. W., and A. R. Williamson. 1971. Cell surveillance model for lymphocyte cooperation. Nature (Lond.). 234:454.

10. Rubin, A. S., and A. H. Coons. 1972. Specific heterologous enhancement of immune responses. III. Partial characterization of a supernatant material with enhancing activity. J. Immunol. 108:1597.

11. Hartman, K. U. 1970. Induction of a hemolysin response in vitro. I. Interaction of cells of bone marrow origin and thymic origin. J. Exp. Med. 132:1267.

12. Miller, J. F. A. P., A. Basten, J. Sprent, and C. Cheers. 1971. Interaction between lymphocytes in immune responses. Cell. Immunol. 2:469.

13. Schrader, J. W. 1973. Specific activation of the bone marrow-derived lymphocyte by antigen presented in a nonmultivalent form: evidence for a two-signal mechanism of triggering. J. Exp. Med. 137:844.

14. Dukor, P., and K. U. Hartman. 1973. Bound C3 as the second signal for B-cell activation. Cell. Immunol. 7:249.

15. Schrader, J. W. 1973. The mechanism of bone-marrow-derived (B) lymphocyte activation. I. Early events in antigen-induced triggering in the presence of polymerized flagellin. Eur. J. Immunol. In press.

16. Schrader, J. W. 1973. The mechanism of bone marrow derived (B) lymphocyte activation. II. A “second signal” for antigen-specific activation is provided by both flagellin and lipopolysaccharide. Eur. J. Immunol. In press.

17. Pantalouris, E. M. 1968. Absence of thymus in a mouse mutant. Nature (Lond.). 217:370.

18. Feldmann, M., H. Wagner, A. Basten, and M. Holmes. 1972. Humoral and cell mediated responses in vitro of spleen cells from mice with thymic aplasia (nude mice). Aust. J. Exp. Biol. Med. Sci. 50:651.

19. Cunningham, A. J., and A. Szenberg. 1968. Further improvements on the plaque technique for detecting single antibody forming cells. Immunology. 14:599.

20. Wagner, H., and M. Feldmann. 1972. Cell mediated immune response in vitro. I. A new system for the generation of cell-mediated cytotoxic activity. Cell. Immunol. 3:405.

21. Unanue, E. R., B. A. Askonas, and A. C. Allison. 1969. A role of macrophages in the stimulation of immune responses by adjuvants. J. Immunol. 103:71.

22. Spitznagel, J. K., and A. C. Allison. 1970. Mode of action of adjuvants: effects on antibody responses to macrophages associated bovine serum albumin. J. Immunol. 104:128.

23. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1971. Carrier function in anti-hapten antibody responses. III. Stimulation of antibody synthesis and
facilitation of hapten-specific secondary antibody responses by graft-versus-host reactions. *J. Exp. Med.* **133**:169.

24. Osbourne, D. P., Jr., and D. H. Katz. 1973. The allogenic effect in inbred mice. III. Unique antigenic structural requirements in the expression of the phenomenon on unprimed populations in vivo. *J. Exp. Med.* **137**:991.

25. Feldmann, M., and A. Basten. 1972. Cell interactions in the immune response in vitro. IV. Comparison of the effects of antigen-specific and allogeneic thymus-derived cell factors. *J. Exp. Med.* **136**:722.

26. Forbes, I. J. 1965. Induction of mitosis in macrophages by endotoxin. *J. Immunol.* **94**:37.

27. Gallily, R. 1973. Uptake and degradation of a synthetic antigen by mouse macrophages containing different levels of lysosomal enzymes. *Adv. Exp. Med. Biol.* **29**:239.

28. Klinman, N. R. 1972. The mechanism of antigen stimulation of primary and secondary clonal precursor cells. *J. Exp. Med.* **136**:241.

29. Katz, D., and B. Benacerraf. 1972. The regulatory influence of activated T-cells on B-cell responses to antigen. *Adv. Immunol.* **15**:1.

30. Vischer, T. L. 1972. Mitogenic factors produced by lymphocyte activation: effect on T- and B-cells. *J. Immunol.* **109**:401.

31. Schrader, J. W. 1973. Evidence for the presence in unimmunized mice of two populations of bone-marrow-derived (B) lymphocytes, defined by differences in adherence properties. *Cell. Immunol.* In press.

32. Kindred, B. 1971. Immunological unresponsiveness of genetically thymusless (nude) mice. *Eur. J. Immunol.* **1**:59.

33. Sela, M. 1972. In Genetic Control of Immune Responsiveness. H. O. McDevitt and M. Landy, editors. Academic Press, New York. 269.

34. Unanue, E. R. 1972. The regulatory role of macrophages in antigenic stimulation. *Adv. Immunol.* **15**:95.

35. Katz, D. H., and E. R. Unanue. 1973. Critical role of determinant presentation in the induction of specific responses in immunocompetent lymphocytes. *J. Exp. Med.* **137**:967.

36. Bach, F. H., B. J. Alter, S. Suliday, D. C. Zoschke, and M. Janis. 1970. Lymphocyte reactivity in vitro. II. Soluble reconstituting factor permitting response of purified lymphocytes. *Cell. Immunol.* **1**:219.

37. Gery, L., and B. N. Waksman. 1972. Potentiation of the T lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). *J. Exp. Med.* **136**:143.

38. Unanue, E. R. 1970. Thymus dependency of the immune response to KLH: an evaluation of the role of macrophages in thymectomized mice. *J. Immunol.* **106**:1339.