

THE IMMUNE RESPONSE AGAINST MYELIN BASIC
PROTEIN IN TWO STRAINS OF RAT WITH
DIFFERENT GENETIC CAPACITY TO DEVELOP
EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS*

By DALE E. McFARLIN‡, SHARON CHUNG-LING HSU, SUSAN B. SLEMENDA, FRANK
C-H. CHOU, AND ROBERT F. KIBLER

(From the Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30309)

Experimental allergic encephalomyelitis (EAE)¹ is produced by injecting whole central nervous system tissue or the myelin basic protein (BP), emulsified in complete Freund's adjuvant (CFA), into genetically susceptible animals. In rats, the major encephalitogenic determinant of BP is contained in a 43 amino acid fragment, residues 45-87 (Fig. 1), of the myelin basic protein (1). Recently, it has been demonstrated that the capacity to develop EAE in rats is under control of a gene, designated by Williams and Moore (2) as the *Ir-EAE* gene, which is linked to the major histocompatibility locus in the rat (2, 3). However, the mechanisms responsible for genetic control are unknown. In the present study both cell-mediated immunity and antibody formation to the intact myelin basic protein and to the 43 residue encephalitogenic fragment (EF) were investigated in the highly susceptible Lewis (Le) rats, which are homozygous for the *Ir-EAE* gene, and in the resistant Brown Norway (BN) rats which lack the *Ir-EAE* gene. The findings indicate that only Le rats have the capacity to mount a cell-mediated immune response against the EF, but that both strains of rats have the capacity to develop antibody against other portions of the BP molecule. Antibody production is greater in the Le rat suggesting that the encephalitogenic determinant, recognized by T cells of the Le rat, may function as a helper determinant in the production of antibody.

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¹ Abbreviations used in this paper: ABC, molar-binding antigen capacity; BN, Brown Norway; BP, basic protein; EF, encephalitogenic fragment; EAE, experimental allergic encephalomyelitis; GPBP, guinea pig basic protein; IFA, incomplete Freund's adjuvant; Le, Lewis; LNC, lymph node cells; MIF, migration inhibitory factor; PEC, peritoneal exudate cells; RIEP, radioimmuno-electrophoresis.

with the same buffer. The breakthrough fractions were pooled and concentrated. The column was then eluted with starting buffer to which sufficient NaCl had been added to increase the conductivity to 2.0 mmho. The fractions were pooled and concentrated. Both pools were analyzed by immunoelectrophoresis using rabbit anti-whole rat serum (Microbiological Associates). Preparations which showed only IgG were used for immunization of adult New Zealand white rabbits. 2 mg of the rat IgG was emulsified in CFA and injected intradermally at multiple sites along the back. 4 wk later rabbits were boosted intravenously with alum precipitated IgG for 5 successive days for a total of 10 mg (6). 7-10 days following the last boost, the animals were bled.

igM. An euglobulin precipitate was prepared by dialysis of 40 ml of rat serum against multiple changes of 1% boric acid for 72 h. The precipitate was washed in 1% boric acid, suspended in and dialyzed against borate-buffered saline (BBS), and filtered through Sephadex G 200; protein eluting the void volume was pooled and concentrated. Rabbits were injected intradermally with 1 mg emulsified in CFA, and boosted 1 mo later with 1 mg in IFA. 10 days after the last injection, the rabbits were bled. When tested in immunoelectrophoresis the rabbit antiserum showed antibodies against IgM, IgG, and three proteins in the alpha and beta globulin region. Antibodies against IgG were removed by absorption with IgG. The resulting antiserum (no. 4171) was used in RIEP.

Detection of Anti-BP Antibodies

RIEP. Rat sera were subjected to immunoelectrophoresis using one well and two troughs. The rat serum was mixed with an equal vol of [^{131}I]BP (1×10^{-7} M) and added to the central well. After electrophoresis one trough was filled with rabbit antirat IgG, and the other trough with rabbit antiserum no. 4171. After developing, the slides were photographed, washed in BBS for 48 h, washed in water for 1 h, dried and allowed to develop on Kodak X-ray film (Eastman Kodak Co., Rochester, N.Y.) for optimal periods of time.

QUANTITATIVE BINDING. Reactivity of radioiodinated BP with serum from immunized rats was sought by Farr's method (7) using 50% saturated ammonium sulfate to precipitate antigen-antibody complexes. Although increased binding was found by immune rat sera, the binding of normal rat sera was 20-25%, which is higher than with other antigens. RIEP analysis of normal rat serum with [^{131}I]BP showed binding to two alpha globulins which was presumably responsible for the increased background (6). RIEP analysis of sera from rats hyperimmunized with GPBP also showed binding to the two alpha globulins as well as binding to IgG. Therefore, a radioimmunoassay was developed using the rabbit antirat IgG described above to precipitate the antigen-antibody complexes.

10 μl of rat serum were added to disposable Falcon plastic tubes (no. 2052, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) followed by 100 μl of 0.2 M borate buffer containing 0.05% normal rat serum, 10^{-8} M GPBP and an amount of [^{125}I]BP sufficient to give approximately 10^8 cpm. The samples were incubated at 37°C for 1 h; 100 μl of specific rabbit antirat IgG serum containing 3 mg antibody/ml were added and incubation continued at 37°C for 30 min and then at 4°C for 2 h. The tubes were centrifuged at 1,750 g at 4°C for 90 min, the supernates aspirated, and precipitates counted. In each determination controls included assay of normal rat serum and the standard rat anti-BP antiserum. The percent binding and the molar binding antigen capacity (ABC) were calculated by the method of Brownstone et al. (9). In the results reported below, the percentage of binding by the standard rat anti-BP serum was reproducible within 5% or less from experiment to experiment.

Studies of Cell-Mediated Immunity

SKIN TEST. Skin tests were performed on a shaved area of the abdomen. Preliminary studies, as reported by others (2, 10), revealed that skin tests diminish and may even become negative as EAE develops. Therefore, animals were studied on the 9th day after immunization. Groups of five rats of each type were injected intradermally with either 50 μg GPBP or an equimolar amount of EF (12.5 μg) dissolved in 0.1 ml of saline. The diameter of the erythema and induration was measured at 1, 4, 24, and 48 h after injection. In some experiments the area of induration was excised at 48 h, fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin.

PREPARATION OF LYMPH NODE CELL SUSPENSION. At various intervals after immunization, rats were killed by cervical dislocation and the draining lymph nodes were removed, dissected from the surrounding connective tissue, and teased in cold PBS. The suspension of LNC was filtered through sterile gauze, washed in sterile PBS, counted and the viability determined by 1% trypan blue exclusion.

LYMPHOCYTE STIMULATION. LNC were resuspended in medium 199 containing 20% fresh, syngeneic rat serum at a concentration of 2×10^6 cells per ml and the appropriate concentration of antigen or mitogen. In preliminary studies, 50 μg of basic protein, 12.5 μg of EF and 2.5 μg of phytohemagglutinin (MR 68, Wellcome Research Laboratories, Beckenham, England) per ml gave the maximum responses, respectively. LNC from each animal were cultured in the absence of antigen and at the above concentrations of BP, EF, and PHA. A microculture system using plastic microtiter (Falcon no. 3040) was employed; 0.2 ml of the cell suspension was added to each well. Quadruplicate cultures were established for each variable. The microtiter plates containing the lymphocyte cultures were incubated at 37°C in 5% CO₂ and harvested after 24, 48, and 72 h. 4 h before harvest, 10 μl of medium 199 containing 20 $\mu\text{Ci/ml}$ of NET-027 [*methyl*-³H]thymidine (spec act 6.7 Ci/mmol, New England Nuclear) were added to each well. The cultures were harvested using an automatic cell harvester (Mash II apparatus, Microbiological Associates). Cells were collected on filters which were dried and added to scintillation counting vials containing 12 ml of Nuclear-Chicago counting solution (Nuclear Chicago Corp., Des Plaines, Ill.) and counted in a Packard liquid scintillation counter (Packard Instrument Co., Downers Grove, Pa.). A stimulation index was calculated by dividing the mean cpm of stimulated cultures by the mean cpm of unstimulated cultures. An index of 1.5 or greater was regarded as significant. In individual experiments the mean responses and standard deviation for groups of five rats of each type were calculated and compared using a Student's *t* test.

MACROPHAGE MIGRATION. Peritoneal exudate cells (PEC) were obtained by injecting male syngeneic rats with 10 ml of sterilized paraffin oil. 5 days later the rats were exsanguinated; the peritoneal cavities were opened aseptically and lavaged with sterile PBS containing 10 U of heparin (Pan Heparin, Abbott) per ml. The PEC were washed three times in PBS and adjusted to a concentration of 2×10^6 cells/ml. To this suspension was added an equal vol of 2×10^7 LNC/ml obtained from the draining nodes of individual rats. Aliquots of PEC and LNC were incubated both in the absence and the presence of antigen. The same concentrations of antigen used in lymphocyte transformation were employed. The macrophage migration was performed by radial diffusion in agarose by a modification of the method developed by Gaines et al. (11). Plastic tissue culture dishes (Falcon no. 3002) containing 0.67% agarose in medium 199 supplemented with 10% fresh syngeneic rat serum was prepared immediately before use. Wells were cut in the agarose, filled with the cell mixtures and incubated at 37°C for 24 h in 5% CO₂. The preparations were then fixed in 10% acetic acid, stained with Ponceau S and the agarose removed. The surface was allowed to dry, the patterns projected on graph paper and the areas of cell migration measured. A migration inhibition index was calculated by dividing the area of migration of cells incubated in the presence of antigen by the area of migration of cells incubated in the absence of antigen, multiplied by 100. An index below 80% was felt to represent significant inhibition. In a few studies, indirect macrophage migration was performed by adding supernates from LNC, cultured either in the presence or in the absence of antigen, to PEC prepared as described above.

Results

Skin Test. The skin test results 9 days after original challenge are shown in Table I. Immediate skin reactions did not occur. Both BN and Le produced positive delayed reactions when challenged with GPBP. The endurance was maximal between 24 and 48 h. Microscopically, the site had the typical appearance of a delayed skin reaction with infiltration of mononuclear cells. In Le rats, the reactions were more intense, both grossly and microscopically. Le rats also developed definite delayed reactions to the EF; however, the magnitude of the reactions against EF was always less than that seen against the intact GPBP. BN rats showed neither gross nor histological evidence of a delayed skin reaction to the EF. In other experiments in which BN and Le rats were skin tested with either GPBP or EF 4 wk after initial challenge, the findings were essentially the same.

Lymphocyte Stimulation. In pilot studies Le and BN rats were sacrificed at

TABLE I
Results of Skin Testing

Antigen	Le rats		BN rats	
	No. positive/ no. tested	Mean* induration	No. positive/ no. tested	Mean induration
		<i>mm</i>		<i>mm</i>
GPBP	5/5	5.8	4/4	3.0
EF	5/5	3.2	0/5‡	—

Groups of BN and Le rats challenged with 50 μ g GPBP were skin tested with either GPBP or EF 9 days after challenge. There was no reaction in the first 4 h; the results after 48 h are shown. All skin test sites were biopsied and studied microscopically.

* Diameter of induration in mm at 48 h.

‡ Negative both grossly and microscopically.

various intervals after challenge and cells from the draining lymph nodes were tested for their capacity to undergo *in vitro* stimulation when cultured with either GPBP or EF. LNC from both BN and Le rats immunized with CFA alone did not exhibit any significant lymphocyte stimulation to BP. In Le rats challenge with BP, the maximal response (SI) was observed at 11-12 days after challenge, which coincided with the onset of clinical symptoms. The response in BN rats challenged with BP, although considerably less than that seen in Le rats, was also maximal at 11-12 days. In subsequent experiments, groups of five Le and five BN rats were sacrificed at day 11 and the *in vitro* response of the LNC from each rat measured. The results of one experiment are shown in Table II. LNC from Le rats showed a definite incorporation of [3 H]thymidine when exposed to the intact BP; furthermore, the mean response to the 43 residue EF was indistinguishable from that of the intact protein. In contrast BN rats showed only marginal response to the BP (SI 2.0 ± 0.7) and no significant response to the EF.

Macrophage Migration. The capacity of LNC from five Le and five BN rats to inhibit the migration of PEC was also assessed 11 days after challenge. The results are shown in Table II. Le LNC produced significant inhibition of PEC migration when incubated with either the intact BP or with the EF. In contrast, BN LNC did not produce significant MIF to either of these proteins. In order to determine if a soluble factor was responsible for the macrophage migration inhibition an indirect MIF assay was employed. Supernates from Le LNC cultures incubated either with or without BP for 48 h were added to the PEC preparation and assayed for MIF. There was significantly less migration with the supernate obtained from LNC incubated with BP, indicating that a soluble factor was responsible for the inhibition of migration.

Antibody Formation. After challenge with 50 μ g of GPBP rats were exsanguinated by cardiac puncture at various intervals; the sera were tested for anti-BP protein antibody by radioimmunoassay (Fig. 2). In the Le rats significant binding (ABC above 1.0) was not detected until day 21, and a peak mean ABC of 4.2 was observed at day 32. In contrast, sera from BN rats did not show significant binding. After boosting with 250 μ g GPBP on day 34, the ABC

TABLE II
In Vitro Lymphocyte Responses

	Le	BN	P
Lymphocyte transformation (stimulation index)			
BP	6.4 ± 2.8	2.0 ± 0.7	<0.01
EF	6.5 ± 2.5	1.2 ± 0.3	<0.01
PHA	22.6 ± 11.9	17.7 ± 3.3	NS
Macrophage migration (%)			
BP	64. ± 5.0	95. ± 2.0	0.001
EF	66. ± 5.0	98. ± 2.0	0.001

Lymph node cells from 5 BN and 5 Le rats challenged with 50 μ g GPBP were tested for their capacity to undergo lymphocyte transformation and to inhibit the migration of PEC. The mean responses to GPBP and EF are shown.

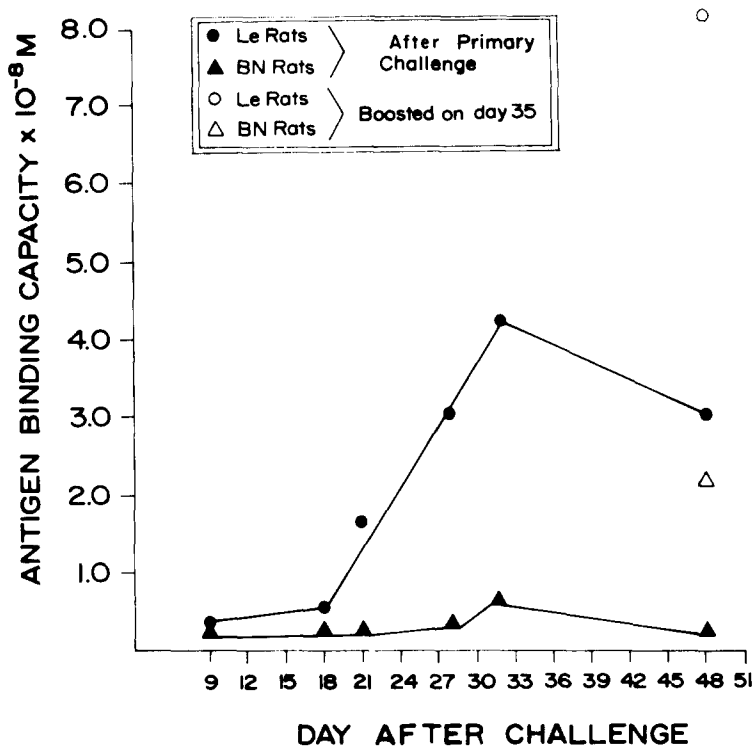


FIG. 2. The mean anti-BP antibody responses in BN and Le rats challenged with 50 μ g GPBP on day 0 and boosted with 250 μ g GPBP on day 35. Each point is the mean response of groups of three to five animals.

rose to 8.1 in Le and to 2.2 in BN rats. In a subsequent experiment (Table III) Le and BN rats were bled via the tail vein 32 days after challenge, boosted on day 34, and rebled on day 48. At the time of the first bleeding Le serum showed a mean ABC of 4.1 while the mean ABC of BN sera was insignificant. After the second

TABLE III
Antibody Formation in Rats-Antigen-Binding Capacity $\times 10^{-8} M$

Day 32*	Day 48‡
Le 4.1 ± 1.4	7.6 ± 1.4
BN 0.62 ± 0.4	1.8 ± 0.63

Groups of Le and BN rats were challenged with 50 μg GPBP and the primary antibody response measured on day 32. The rats were boosted with 250 μg on day 35 and the secondary antibody response measured on day 48. The mean response \pm one standard deviation for groups of five rats are shown.

* After challenge with 50 μg GPBP in FCA.

‡ Secondary response following boost with 250 μg in ICA day 35.

injection on day 34 there was a rise in ABC in both strains; to 7.6 in Le rats and 1.8 in BN rats.

Sera were also studied by RIEP using [^{131}I]BP. In all Le sera with significant ABC there was reactivity between the BP and IgG and in an occasional serum there was also reactivity with IgM. In addition, it is noteworthy that sera from some BN rats obtained 21 and 28 days after challenge showed faint binding with IgG, indicating that BN rats produce small amounts of anti-BP antibody undetectable by the radioimmunoassay. However, no serum obtained before day 21 from rats of either strain showed binding of antigen by RIEP. All sera from boosted animals of both strains showed reactivity with IgG and in an occasional serum from both BN and Le rats there was also binding to IgM.

Antibody Binding Site. Because the in vitro studies of cell-mediated immunity described above indicated the reaction to EF was equal to that of the entire BP molecule, it seemed important to determine if the antibody was directed at this portion of the molecule. This question was approached in the following manner. Radioimmunoassay of rat anti-BP was performed by serially diluting hyperimmune rat serum in three ways: with diluting buffer, diluting buffer containing 100 molar excess of BP, and diluting buffer containing 100 molar excess of EF. The results of a representative experiment with a Le anti-BP serum are shown in Fig. 3. Complete inhibition of binding was obtained with 100 molar excess BP, but none was obtained with 100 molar excess EF, even at the greatest dilution of the serum. When BN anti-BP anti sera were studied in the same manner comparable results were obtained. These inhibition studies indicated that EF does not react with anti-BP antibody which likely is directed at a site other than the EF.

Discussion

In previous studies we have demonstrated in the Le rat that GPBP is a more potent encephalitogen than either rat BP or bovine BP; and that the encephalitogenic potency of the 43 residue EF is identical to that of the respective intact BP when administered on an equimolar basis (1). In the present study, Le rats challenged with GPBP, under the conditions routinely employed to produce EAE

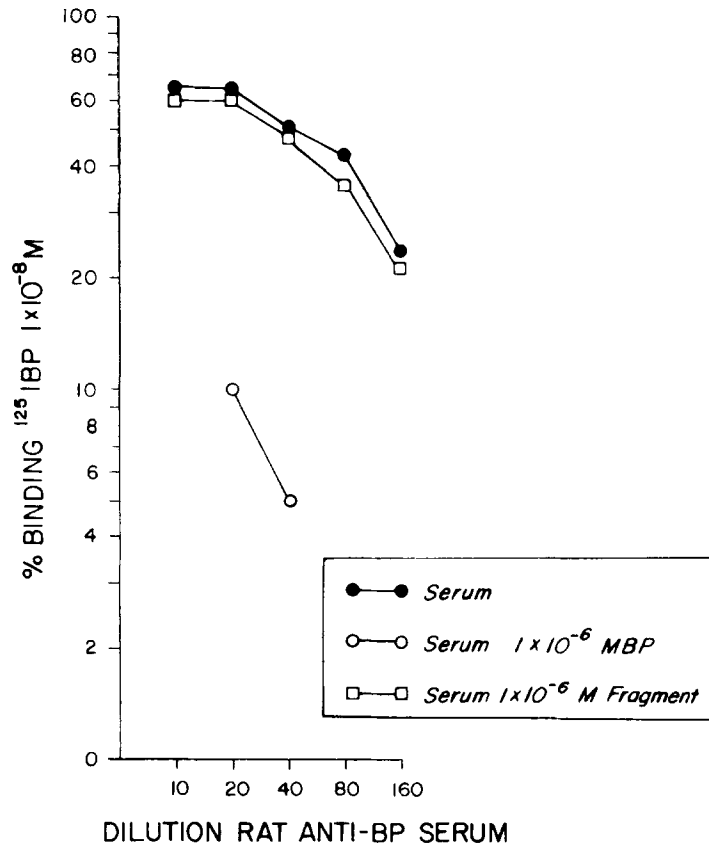


FIG. 3. Titration of Le rat serum with anti-BP antibody. When diluted in 100 molar excess of intact GPBP there was inhibition of binding; however, no inhibition was observed with 100 molar excess of EF.

in this strain, developed delayed skin tests to both GPBP and to the GPEF and cells from their draining lymph nodes incorporated [^3H]thymidine and produced MIF when incubated in vitro with either antigen. BN rats which do not develop EAE, developed positive delayed skin tests against GPBP, but the magnitude of the response was considerably less than that seen in Le rats. The results of in vitro tests with GPBP were consistent with these observations. No skin response was seen in BN rats to the EF, however, and cells from draining nodes did not incorporate [^3H]thymidine or produce MIF when incubated with this fragment in vitro. The cell-mediated immune response to GPBP which BN rats developed appears, therefore, to be against a portion of the molecule other than the EF.

In Le rats, the demonstration of a delayed skin test and the capacity to develop in vitro evidence of cell-mediated immunity to the EF are consistent with a T-cell response against this determinant. These findings, coupled with data demonstrating genetic control of the capacity to develop EAE in rats (2, 3), support the concept that the so called *Ir*-EAE gene functions by regulation of the T-cell response against the EF portion of the GPBP molecule.

Both strains of rat were shown to develop antibody against GPBP by

radioimmunoassay. Antigen binding in this system is related to both antibody concentration and antibody affinity (12). These properties are best reflected by expressing the data as ABC which has been used in other antibody systems (7, 9, 13). Using this approach, it was demonstrated that Le rats developed a small but detectable primary response to GPBP. BN rats did not show detectable antibody by radioimmunoassay but probably developed in minimal response as indicated by the results of RIEP. After secondary challenge, both strains of rat showed a rise in ABC; however, this was far greater in the Le strain of rat. The precise determinant(s) against which antibody is directed in the rat is not known. We elected to use an inhibition assay, (rather than the direct radioimmunoassay) to determine whether antibody was directed at the EF portion of the molecule since iodination of the single tryosine of EF (Fig. 1) might alter its capacity to bind in this system. The inhibition studies indicate that the antibody in both strains of rat is directed at some determinant(s) other than the EF. Thus, as has been demonstrated with glucagon in the mouse (14), the T cells of the Le rat react primarily with one portion of the GPBP while B cells react with another portion. Since both strains of rats produce antibody, it would appear that recognition of the antibody determinant(s) is controlled by a gene other than the *Ir-EAE* gene.

Studies of carrier-hapten systems have shown that although T cells and B cells recognize different determinants during the production of antibody, the T-cell determinant functions as a helper determinant in the production of antibody (15). It is possible to interpret our findings according to this system and to postulate that the EF contains a helper determinant for the production of antibody in Le rats. When viewed in this fashion, BN rats would have B cells which respond to the antibody determinant but which produce only minimal amounts of antibody because they lack T cells with the capacity to recognize the helper determinant. Boosting with antigen results in greater proliferation of the B cells and a detectable secondary response.

Summary

After challenge with guinea pig basic protein (GPBP) Lewis (Le) rats, which are homozygous for the immune response experimental allergic encephalomyelitis (*Ir-EAE*) gene, developed positive delayed skin tests against GPBP and the 43 residue encephalitogenic fragment (EF); in addition, Le rat lymph node cells (LNC) were stimulated and produced migration inhibitory factor (MIF) when incubated in vitro with these antigens. In contrast Brown Norway (BN) rats, which lack the *Ir-EAE* gene, did not develop delayed skin tests to EF and their LNC were not stimulated and did not produce MIF when incubated in vitro with EF. These observations indicate that the *Ir-EAE* gene controls a T-cell response against the EF.

Le rats produced measurable anti-BP antibody by radioimmunoassay after primary challenge. Although no antibody was detectable in BN rats by radioimmunoassay, radioimmunoelectrophoresis indicated that a small amount of antibody was formed after primary immunization. After boosting intraperitoneally, both strains of rat exhibited a rise in anti-BP antibody; which was greater

in Le rats. In both strains of rat the anti-BP antibody reacted with a portion of the molecule other than the EF. Since EF primarily evokes a T cell response, it is suggested that the EF portion of the BP molecule may contain a helper determinant in antibody production.

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