

SUPPRESSION OR AUGMENTATION OF THE ANTIHAPTEN
RESPONSE IN MICE BY ANTIBODIES OF DIFFERENT
SPECIFICITIES*

BY G. HAUGHTON‡ AND O. MÄKELÄ

(From the Department of Serology and Bacteriology, University of Helsinki,
Helsinki 29, Finland)

(Received for publication 21 March 1973)

The ability of humoral products of the immune response to regulate the response is well known (1). Serum from immune individuals, passively administered to normal recipients, can suppress both the rejection of grafted tissues (2) and the antibody response to homologous antigen (3). The possibility that the two phenomena were distinct emerged with the realization that two different populations of lymphoid cells were involved. Thymus-independent, bone marrow-derived lymphocytes (B cells) are the line of cells that can ultimately mature to antibody production. The lymphocytes responsible for cellular immunity and that also may subservise a helper function in humoral antibody production (T cells) require the influence of the thymus for their maturation (4, 5). The production of antihapten antibody in response to immunization with hapten-carrier conjugates involves cooperation of T cells and B cells (6, 7), the former reacting with carrier specificities and the latter with the haptenic determinant, and may also involve macrophages (8-10). Any or all of these may be susceptible to suppression by humoral factors.

Several workers have found evidence that hyperimmune serum can inhibit the processing by macrophages of heterologous erythrocytes to an immunogenic form (11-13). Suppression of B cell maturation and proliferation by antibody probably represents direct competition for antigen between B cell receptors and antibody (14), and it is reported that this may occur without concomitant suppression of T cells (15). However, suppression of T cell development and/or function by humoral factors occurs in both natural and induced enhancement of antigenically foreign tissues (2, 16-18).

In addition to immunosuppression induced by humoral factors, it has been established that relatively small doses of passively administered immune serum may cause augmentation of antibody production. This effect has been variously ascribed to IgM antibody presumably directed against the haptenic determinant (19), or to antibody directed against new antigenic determinants introduced into the carrier molecule during the process of haptening (20-22).

The series of experiments reported in this paper were designed to elucidate the

* Supported by the National Research Council for Medical Sciences, Helsinki, Finland, and by a Research Career Development Award from the U. S. Public Health Service (5K03 CA38664).

‡ Present address: Department of Bacteriology and Immunology, University of North Carolina Medical School, Chapel Hill, N. C. 27514.

regulatory role of antibody. We have employed antisera raised against a family of related haptens coupled to non-cross-reacting protein carriers both before and after absorption with insoluble immunoadsorbents. We have measured the effect of passive administration of these sera on the level of the antihapten response to a standard challenge with hapten-carrier conjugate.

AnticARRIER sera cause a modest degree of immunosuppression when administered in high doses. Antihapten sera are strongly immunosuppressive in high doses but augment the response in low doses. Suppression and augmentation are due to distinct populations of antibodies, suppression being a function of anti-hapten antibodies and augmentation a function of antibodies directed against specificities introduced into any of the protein carriers as a result of conjugation with any of the family of haptens.

Materials and Methods

Mice.—Two strains were used. Donors of the suppressing anti-NIP-CG¹ antiserum were outbred white mice. For other purposes we used (CBA × C57)F₁ bred in this laboratory. The origin of the CBA parent was the National Institute for Medical Research, London, and the origin of the C57BL/6 the Jackson Laboratory, Bar Harbor, Maine.

Antigens and Haptens.—The haptens NIP (3-nitro-4-hydroxy-5-iodophenylacetic acid), NP (3-nitro-4-hydroxyphenylacetic acid), or HOP (4-hydroxyphenylacetic acid) were coupled to chicken globulin (CG), bovine serum albumin (BSA), or ovalbumin (OA) as previously described (23).

Preparation of Immunosuppressive Antisera.—An emulsion of complete Freund's adjuvant (CFA) with an equal volume of NIP₁₂-CG (1 mg/ml in saline) was prepared. Each of 30 outbred white mice received four injections of 50 μ l of this emulsion, one in each axillary and inguinal region. 3 wk later this treatment was repeated and after a further 6 wk each mouse was injected intraperitoneally (i.p.) with 300 μ g of alum-precipitated NIP₁₂-CG in 0.3 ml of saline. The mice were bled from the retro-orbital sinus 10 days after the last injection, the serum was separated, and after a small sample had been saved from each mouse the bulk was pooled for storage in small aliquots at -20°C .

Antisera to NIP₄-OA and to CG were prepared in a similar fashion except that the mice were (CBA × C57)F₁ and the first injection in CFA was omitted. All mice used for preparation of these sera were females.

Immunoadsorbents.—Agarose (Sephacrose 4B; Pharmacia, Uppsala, Sweden) was activated according to Porath et al. (24). The beads were washed twice with 10 vol of water and packed Sepharose (1 vol) was suspended in an equal volume of water. The mixture was placed on a magnetic stirrer at room temperature and 6 vol of 1% CNBr in water was added. The mixture was incubated for 1 min at pH 4-5 and the electrode of a pH meter was put into the mixture. Then enough 1 N NaOH was added to keep the pH at 10.5 ± 0.5 . When 0.4 vol of NaOH had been added the mixture was poured into 60 vol of 0.1 M NaHCO₃ and the beads were washed in a Büchner funnel with 120 vol of 0.1 M NaHCO₃.

Activated Sepharose was suspended into 2 vol of 0.1 M NaHCO₃ and 600 mg of protein was added. The mixture was incubated overnight at 4°C. The beads were washed in a sintered glass funnel with 10 vol of borate buffer, 10 vol of 8 M urea, 10 vol of citrate buffer pH 2.6,

¹ *Abbreviations used in this paper:* ABC, antigen-binding capacity; ABC₃₀, reciprocal of serum dilution at which 30% of 10^{-8} M free hapten is bound; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CG, chicken globulin; EDA, ethylenediamine; HOP, 4-hydroxyphenylacetic acid; NIP, 3-nitro-4-hydroxy-5-iodophenylacetic acid; NP, 3-nitro-4-hydroxyphenylacetic acid; OA, ovalbumin.

10 vol of carbonate buffer pH 10, 10 vol of saline, and 10 vol of 0.05 M ethanolamine in borate buffer. The first five steps were repeated.

For the preparation of hapten-immunoabsorbents the procedure was the above except that instead of protein, 75 mg of NIP-ethylenediamine (NIP-EDA) was added. NIP-EDA was prepared according to Hoffman et al. (25).

The two haptens used in the immunoabsorbents, HOP and NIP, are slightly cross-reactive but both reciprocal K_{rel} values (HOP vs. anti-NIP and NIP vs. anti-HOP) are too low (<0.0001) to be measured accurately (O. Mäkelä, unpublished experiments).

Standard Experimental Design.—All mice used were (CBA \times C57) F_1 hybrids between 12 and 30 wk of age. Within any single experiment all mice were of the same sex and differed in age by not more than 5 wk.

Groups of five mice were injected i.p. with 0.2 ml of appropriately diluted antiserum in saline so that each mouse received from 0.01 to 100 μ l of original serum. Between 16 h and 24 h later these mice, plus a group of controls that had been injected with saline, received 50 μ g of alum-precipitated NIP $_{12}$ -CG i.p. The mice were bled from the retro-orbital sinus under light ether anesthesia 11 and 19 and in some cases 42 days after antigen. The sera were titrated individually to determine their antigen-binding capacity (ABC) for 125 I-labeled NIP-cap (26). Hapten was present in the mixtures with serum dilutions at a final concentration of 10^{-8} M. The data used are observed values for ABC when 30% of the hapten was bound and are presented as the geometric mean value of each group \pm the standard error. The significance of difference between groups was determined by Student's t test.

RESULTS

Suppression of the Primary Response to NIP $_{12}$ -CG by Homologous Antiserum.—Various doses of hyperimmune anti-NIP $_{12}$ -CG ranging from 0.1 to 10 μ l were given to groups of five (CBA \times C57) F_1 σ^7 mice, which were then challenged with 50 μ g of alum-precipitated homologous antigen. Production of NIP-binding antibody was followed for 6 wk. Data are presented in Figs. 1

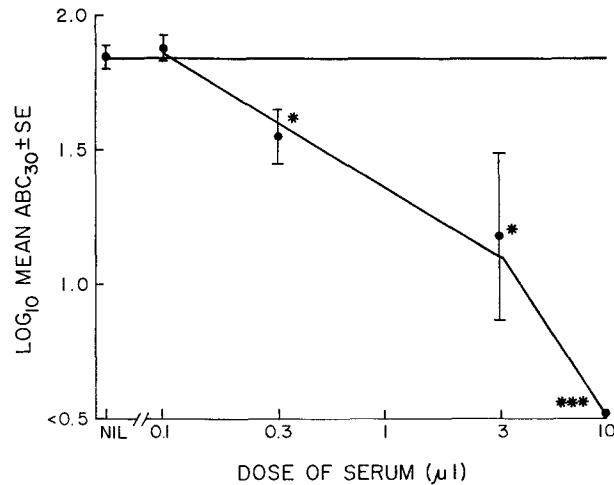


FIG. 1. Suppression of the anti-NIP response to 50 μ g of alum-precipitated NIP $_{12}$ -CG by various doses of hyperimmune homologous antiserum given 16 h before challenge. $\log_{10} ABC_{30}$ of the injected antiserum was 4.08. Data are from groups of five (CBA \times C57) F_1 σ^7 mice bled 20 days after antigen injection. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

and 2. A dose of $0.3 \mu\text{l}$ of antiserum or greater caused a significant degree of immunosuppression. The immunosuppressive effect was greater early in the response than late. Recipients of $10 \mu\text{l}$ of antiserum (the highest dose tested) did not produce detectable levels of antibody during the first 20 days, but by 42 days they had caught up to within about 50% of control levels.

The ABC_{30} of the hyperimmune serum used for immunosuppression was $1.2 \times 10^{-4} \text{ M}$. The dose of antigen used contained approximately 3.3×10^{-9}

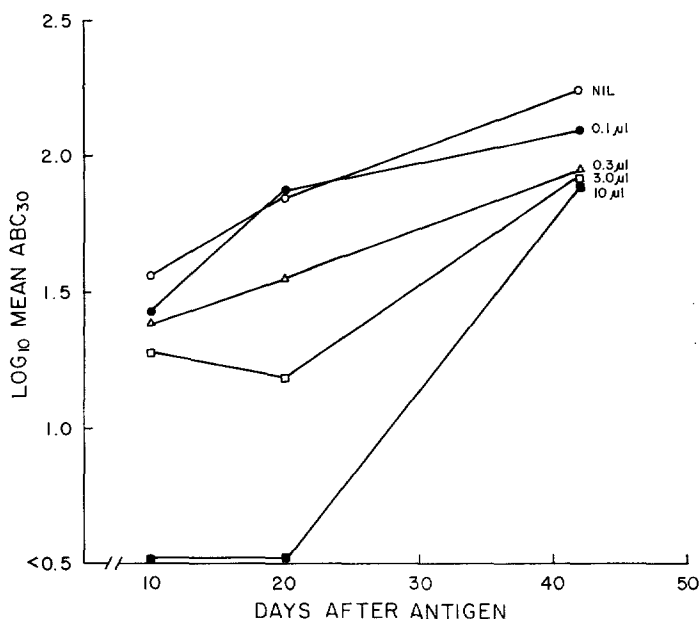


FIG. 2. Production of NIP-binding antibody after injection of various doses of anti-NIP-CG antiserum and challenge with $50 \mu\text{g}$ of alum-precipitated $\text{NIP}_{12}\text{-CG}$. For details see Fig. 1.

mol of NIP and thus a dose of $0.3 \mu\text{l}$ of antiserum contained only sufficient antibody to bind about 1% of the administered hapten.

From this experiment, it could not be determined whether immunosuppression was a function of antihapten antibody, presumably operating at the B cell level, or of anticarrier, presumably operating on the helper T cells.

Influence of Anticarrier and Antihapten Sera on the Primary Response to $\text{NIP}_{12}\text{-CG}$.—Various doses of hyperimmune serum prepared against CG or $\text{NIP}_4\text{-OA}$ were administered to groups of five (CBA \times C57)F₁ ♀ mice, which were then challenged with $50 \mu\text{g}$ of alum-precipitated $\text{NIP}_{12}\text{-CG}$. Production of NIP-binding antibody was followed. Data from the mice treated with anticarrier and with antihapten are presented in Figs. 3 and 4 and Figs. 5 and 6, respectively. High doses of anti-CG (10 and $100 \mu\text{l}$) caused a modest but statis-

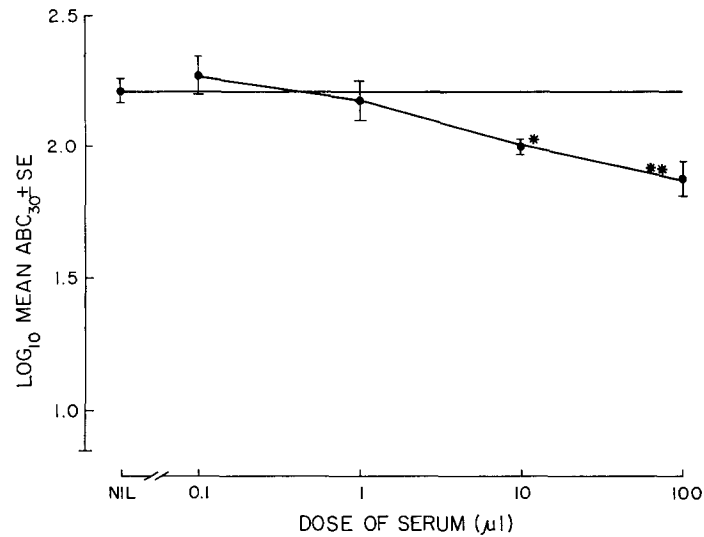


FIG. 3. Influence of anticarrier serum on the primary anti-NIP response to 50 μ g of alum-precipitated NIP₁₂-CG. Groups of five (CBA \times C57) F₁ φ mice were given various doses of hyperimmune anti-CG 16 h before antigen and bled 19 days later. The probability that the observed differences between experimental and control groups is due to chance is indicated by asterisks as in Fig. 1.

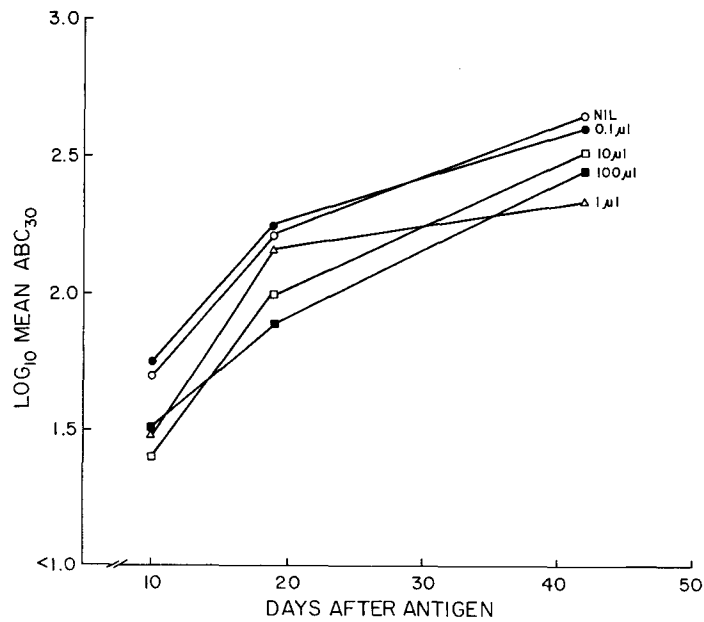


FIG. 4. Production of NIP-binding antibody after injection of various doses of anti-CG serum and challenge with 50 μ g of alum-precipitated NIP₁₂-CG. For details see Fig. 3.

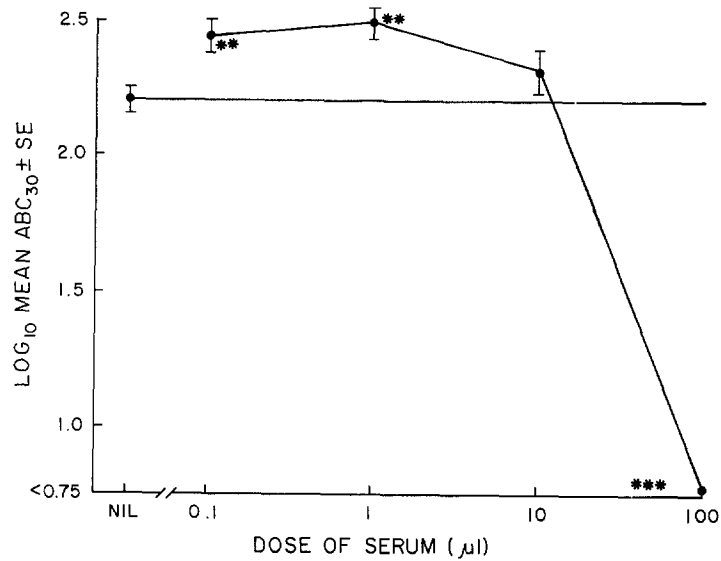


FIG. 5. Influence of antihapten serum on the primary anti-NIP response to 50 μ g of alum-precipitated NIP₁₂-CG. Groups of five (CBA \times C57)F₁ \varnothing mice were given various doses of hyperimmune anti-NIP₄-OA 16 h before antigen and bled 19 days later. The probability that the observed differences between experimental and control groups is due to chance is indicated by asterisks as in Fig. 1.

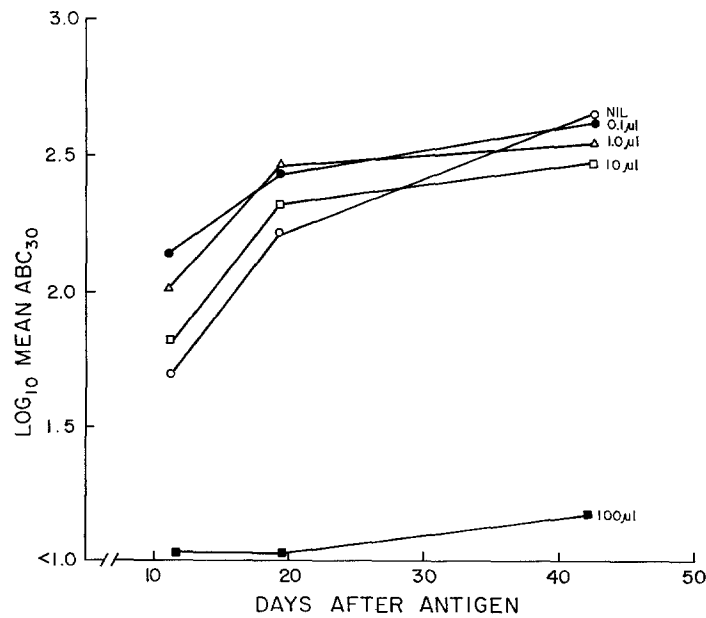


FIG. 6. Production of NIP-binding antibody after injection of various doses of anti-NIP₄-OA serum and challenge with 50 μ g of alum-precipitated NIP₁₂-CG. For details see Fig. 5.

tically significant degree of immunosuppression. By 19 days after antigen these doses of antiserum caused about 35 and 60% reduction, respectively, in anti-NIP antibody below control levels. Lower doses of anti-CG had no discernible effect.

By contrast, a high dose (100 μ l) of anti-NIP₄-OA serum suppressed the anti-NIP response at 19 days by about 99% while low doses (0.1 or 1.0 μ l) increased the response about twofold. Both suppression and elevation of antibody production were most pronounced early in the response. At 11 and 19 days after antigen, both low doses caused significant elevation of the response and 100 μ l caused almost total suppression. By 42 days after antigen, the group treated with 100 μ l of serum were no longer fully suppressed and the groups treated with lower doses had antibody titers similar to those in control mice.

Influence of Absorbed Antisera on the Primary Response to NIP₁₂-CG.—The question of whether both suppression and elevation of the response are due to anti-NIP antibodies present in the serum raised against NIP₄-OA, or whether, as suggested by Rubin (21), elevation was due to antibodies against new antigenic determinants introduced into the protein molecule in the course of haptening was approached by absorbing the anti-NIP₄-OA serum with hapten coupled to insoluble Sepharose.

Anti-NIP₄-OA serum was diluted 1:10 and absorbed with $\frac{1}{4}$ vol of NIP-Sepharose. The mixture was stirred at 4°C for 8 h, the beads were removed by centrifugation, and the supernatant was used. A control sample of serum, which was not absorbed, was kept in diluted form at 4°C for the period of the absorption. Before absorption the ABC₃₀ of the serum was 870 (8.7×10^{-6} M); after absorption it was reduced to less than 0.8 (8×10^{-9} M).

Various doses of the absorbed or unabsorbed serum were given to groups of five (CBA \times C57)F₁ σ mice, which were then challenged with 50 μ g of alum-precipitated NIP₁₂-CG. Anti-NIP titers in the bleedings taken 19 days after antigen are shown in Fig. 7. Removal of more than 99.9% of the NIP-binding antibodies from the serum by absorption with NIP-Sepharose did not diminish the "helper" effect of the anti-NIP₄-OA serum. In fact, at all doses tested, a greater elevation of the response occurred in mice given absorbed serum than in those given unabsorbed serum. The data are consistent with the hypothesis that immunosuppressive activity is a function of hapten-specific antibodies, but that the observed helper effect is due to antibodies directed against a new antigenic determinant introduced into the protein carrier as a result of coupling the hapten to the lysine residues of the protein. This new antigenic determinant would not be created by coupling NIP to carbohydrate and thus antibodies directed against it would not be removed by absorption with NIP-Sepharose.

We argued that if the hypothesis were correct, a similar new antigenic determinant might be created by coupling a different, but chemically related, hapten to a protein carrier. Furthermore, if the new antigenic determinant resulted from the union between hapten and lysine, the nature of the protein carrier

might not be important; a similar new antigenic determinant might occur in any protein coupled through a lysine residue to any hapten of this family.

As a test of this prediction, two equal samples of anti-NIP₄-OA were absorbed: the one with NIP-EDA-Sepharose and the other with HOP-BSA-Sepharose. Equivalent volumes of the two absorbed sera were given to groups of five (CBA × C57)F₁ ♀ mice followed by challenge with 50 μg of alum-precipitated NIP₁₂-CG. The ABC₃₀ of the resulting antisera taken 13 days later are shown in Fig. 8. Absorption with NIP-Sepharose had removed all detectable

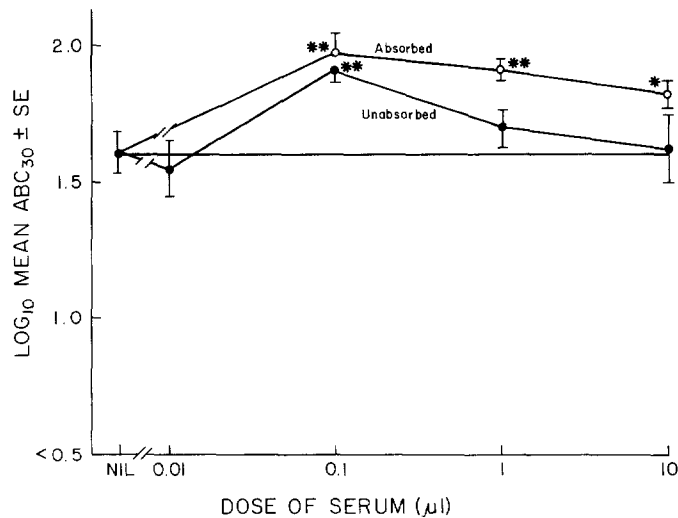


FIG. 7. Effect of removing NIP-binding antibodies from hyperimmune anti-NIP₄-OA on its ability to augment the anti-NIP response to primary challenge with NIP₁₂-CG. A sample of anti-NIP₄-OA was absorbed with NIP-EDA-Sepharose, thereby reducing its NIP-binding capacity by more than 99.9%. Various doses of absorbed or unabsorbed serum were given to groups of five (CBA × C57)F₁ ♂ mice 16 h before challenge with 50 μg of alum-precipitated NIP₁₂-CG. The mice were bled 19 days after antigen. The probability that the observed differences between control and experimental groups is due to chance is indicated by asterisks as in Fig. 1.

suppressing antibody; not even 100 μl caused any suppression. We conclude from this that most of the suppressing antibody in this serum was specific for NIP. This absorption had not reduced the helper effect. Thus the helper factor in the anti-NIP-OA serum probably was not anti-NIP. Absorption with HOP-BSA-Sepharose had removed the helper antibody, and somewhat surprisingly, also the component responsible for potent immunosuppression. We did not expect this because there is very little cross-reaction between NIP and HOP tested by the conventional methods.

To test whether helper antibody was species specific, an antiserum raised

in rabbits against NP-CG was examined. Antihapten antibodies would be poorly cross-reactive with NIP and would not be immunosuppressive. However, the serum likely contained anticarrier antibodies, which could be immunosuppressive, as well as the postulated helper antibodies directed against new antigenic determinants. It was, therefore, absorbed with CG-Sepharose before testing. Data from the test of this absorbed serum are given in Fig. 9. At all

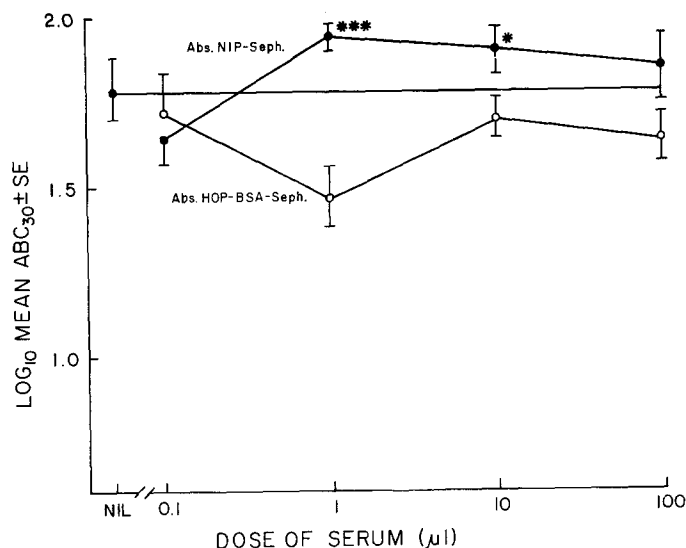


FIG. 8. Effect of removing antibodies against NIP or against postulated new antigenic determinants from anti-NIP₄-OA on its ability to augment the primary anti-NIP response to NIP₁₂-CG. Samples of anti-NIP₄-OA serum were absorbed with NIP-EDA-Sepharose or with HOP-BSA-Sepharose. Various doses of the absorbed sera were given to groups of five (CBA × C57)F₁ ♀ mice 16 h before challenge with 50 μg of alum-precipitated NIP₁₂-CG. The mice were bled 13 days after antigen. The probability that the observed differences between pairs of experimental groups injected with equal doses of the two absorbed sera is due to chance is indicated by asterisks as in Fig. 1.

doses tested, the experimental mice gave an elevated response. At the two lower doses, this elevation was highly significant ($P < 0.01$).

Sex.—During the course of this and related work, we noted that the antibody response observed in groups of control mice varied from one experiment to another. Upon examination it became clear that this variation was sex related, females producing higher antibody titers in response to a standard primary dose of 50 μg of alum-precipitated NIP₁₂-CG. Data from all (CBA × C57)F₁ control mice are presented in Fig. 10. It is clear that female mice give a higher and more prolonged response to the standard primary challenge than do males.

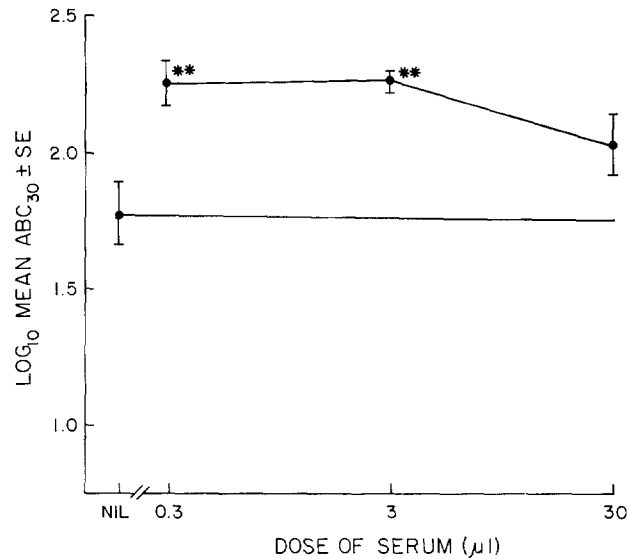


FIG. 9. Augmentation of the primary anti-NIP response of mice to NIP_{12} -CG by various doses of rabbit anti-NP-CG absorbed with CG-Sepharose. Groups of five (CBA \times C57) F_1 \varnothing mice were given various doses of the absorbed serum 16 h before 50 μ g of alum-precipitated NIP_{12} -CG. They were bled 13 days later. The probability that the observed differences between control and experimental groups is due to chance is indicated by asterisks in Fig. 1.

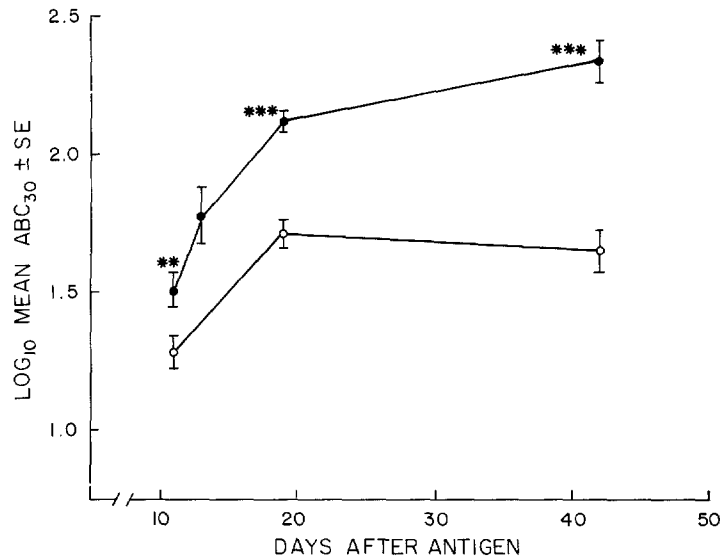


FIG. 10. The influence of sex on the primary anti-NIP response of normal (CBA \times C57) F_1 mice to 50 μ g of alum-precipitated NIP_{12} -CG. Each point represents data from 13–25 females (upper curve) or males. The probability that the difference between the sexes is due to chance is indicated by asterisks as in Fig. 1.

DISCUSSION

Suppression of the immune response by passive antibody operates through competition for antigen between antigen receptors on lymphocyte surfaces and the administered antibody (14). In the case of thymus-dependent antigens, when both T cells and B cells must interact with antigen before antibody production occurs, it is not clear whether competition occurs only at the B cell level, or whether passive antibody may also interfere with the T cell response. In an attempt to attack this question directly, we have administered anti-hapten or anticarrier antibody and observed the effect on the response to challenge with hapten-carrier conjugate. As expected, relatively large doses of hyperimmune antihapten serum caused almost complete suppression of the response, probably by direct competition with B cell receptors. Suppression of the response by homologous antiserum (Fig. 2) or antihapten serum (Fig. 4) was not permanent; some antibody production was seen between 3 and 6 wk after immunization, indicating relatively long persistence of the administered antigen.

Administration of high doses of anticarrier serum caused a moderate but highly significant suppression of the antihapten response. Clearly this effect was not due to direct competition with B cell receptors, but to reaction of antibody with that portion of the antigen that is recognized by helper T cells. One may not conclude that antibody competes directly with T cell receptors, since there is no evidence that anticarrier serum reacts with the same determinants on the molecule as do the T cell receptors. Nonetheless, some mechanism other than direct competition with B cells must be postulated to explain the observed effect.

We have earlier described an inhibitory effect of passive antiserum on antigen handling by macrophages (13), and it is well established that humoral products of the immune response can inhibit T cell functions such as graft rejection (2, 16, 17) and *in vitro* cytotoxicity (18). We conclude that helper function of T cells can similarly be inhibited.

While high doses of antihapten serum caused almost total suppression of the immune response, very much lower doses elevated the response above control levels. Other examples of a positive influence of passive antiserum have been reported (19–22). The effect has been ascribed either to a macromolecular component of the serum, presumed to be IgM, or to antibody directed against nonhapten determinants newly introduced into the carrier molecule as a consequence of the coupling with hapten. The phenomenon that we have observed is more akin to the latter than to the former. IgM anti-NIP seems not to be involved for the following reasons. The serum in question was a product of hyperimmunization, unlikely to contain very much IgM antibody. In accordance with this prediction, the anti-NIP activity of the serum was found in bacteriophage neutralization tests (27) to be highly resistant to mercaptoethanol (O. Mäkelä, unpublished data). The helper activity was not removed

by absorption with NIP-Sepharose, which reduced the ABC for NIP-cap by more than 99.9%. However, the activity could be removed by absorption with HOP-BSA-Sepharose, an antigen whose haptenic group cross-reacts very poorly with NIP.

Antiserum raised in rabbits against NP-CG and absorbed with CG-Sepharose also demonstrated helper activity. The determinant NP also cross-reacts poorly with NIP (28) and the antihapten antibodies did not suppress the anti-NIP response to NIP-CG.

Thus, the characteristics of helper antibody as delineated in this study are as follows. It is not directed against the haptenic group or against the native carrier, but against a new antigenic determinant introduced into the carrier as a result of haptination. The determinant is neither hapten-specific nor carrier-specific but seems to be introduced into any of the three proteins tested (CG, OA, BSA) by the process of haptination through lysine with any hapten of the NIP family. This implies that the determinant in question is adjacent to the introduced hapten and suggests a possible mode of action of the helper antibody. After antigen degradation, only those fragments carrying hapten would express the new antigenic determinant and thus, hapten carrying fragments would be specifically susceptible to aggregation by helper antibody providing a high local concentration of hapten for stimulation of B cells. The question of whether helper antibody can substitute for helper T cells as suggested in the models studied by Rubin (21, 22) or whether the effect is only supplementary cannot be answered from our data.

SUMMARY

We have measured the production by (C57 × CBA) F_1 mice of hapten-binding antibody in response to a standard dose of 50 μ g of alum-precipitated NIP₁₂-CG and the influence on this response of the prior administration of hyperimmune antisera raised against the homologous conjugate, the carrier globulin alone, the hapten conjugated to a non-cross-reactive carrier (NIP₄-OA), or a related hapten (NP) coupled to CG.

The homologous antiserum was strongly immunosuppressive; a dose capable of binding about 1% of the administered hapten caused significant suppression. High doses of anticarrier serum caused significant but modest suppression (about 50%); low doses had no effect. High doses of the serum prepared against NIP₄-OA suppressed the 19 day response by more than 97%, while 100–1,000 times lower doses caused the response to be elevated to about double the control level. The antibodies responsible for immunosuppression could be removed from this serum, as could the NIP-binding antibodies, by absorption with NIP coupled through ethylenediamine to insoluble Sepharose. The ability of this serum to augment the response was not reduced by such absorption. Augmenting antibodies could be removed by absorption with HOP-BSA-Sepharose. Thus, immunosuppression and augmentation are functions of two different

populations of antibody. The former are specific hapten-binding antibodies, the latter seem to be directed against new antigenic determinants created by coupling any of the family of haptens through lysine to protein carriers. In support of this contention, it was observed that rabbit antiserum to NP-CG, after absorption with CG-Sepharose, augmented the response of mice to standard immunization with NIP₁₂-CG.

Female mice produced significantly more NIP-binding antibody than did males.

We are indebted to Mrs. Kaija Rytsy for skillful technical assistance.

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