

STIMULATION OF PRIMARY IN VITRO IgE ANTIBODY
RESPONSES IN CULTURES OF
HUMAN PERIPHERAL MONONUCLEAR CELLS*

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Considerable progress has been made in recent years toward understanding various aspects of regulatory mechanisms that control the IgE antibody system in experimental animals (1-4). The potential clinical implications of the findings made in experimental animals will only be realized after such information has been translated to the human IgE antibody system to determine the extent to which parallel mechanisms operate in man. Development of in vitro technology with human peripheral blood mononuclear cells (MNC)¹ has enabled investigators to analyze mechanisms controlling the synthesis of human IgM, IgG, and IgA (5-10). Fewer studies have been conducted on in vitro synthesis of human IgE largely because of the very small quantities of IgE molecules synthesized by normal individuals both in vivo and secreted into supernatant fluids of cells cultured in vitro. Although Gleich et al. (11) developed a sensitive double antibody radioimmunoassay (RIA) for measuring the small quantities of human IgE found in serum, until recently sufficient quantities of suitable reagents for making such determinations were not generally available.

Recently, studies in several laboratories have been successful in demonstrating biosynthesis of IgE antibody molecules by human lymphocytes in vitro, both spontaneously and after stimulation by suitable polyclonal mitogenic substances (12-17). We have been working to develop a system whereby human lymphocytes could be stimulated not only by polyclonal mitogens but also by specific antigens to develop primary IgE responses in vitro. The ability to induce antigen-specific human IgE responses, we felt, would offer considerable advantages with regard to translating previous findings in experimental animals to the biology of lymphocyte function in man. In this report, we present results demonstrating the ability to stimulate synthesis of human IgE in vitro by exposure to hapten-protein conjugates, either alone or with concomitant exposure to pokeweed mitogen (PWM).

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¹ *Abbreviations used in this paper:* ASC, Ascaris; BSA, bovine serum albumin; DNP, 2,4-dinitrophenyl; FCS, fetal calf serum; GAHE, goat anti-human IgE; GAHG, goat anti-human IgG; KLH, keyhole limpet hemocyanin; MEM, minimum essential medium; MNC, mononuclear cells; NHS, normal human serum; PWM, pokeweed mitogen; RIA, radioimmunoassay.

Materials and Methods

Antigens and Mitogens. Bovine serum albumin (BSA) was purchased from Sigma Chemical Co., St. Louis, Mo., and keyhole limpet hemocyanin (KLH) was purchased from Pacific Bio-Marine Laboratories, Inc., Venice, Calif. Ascaris proteins (ASC), extracted from *Ascaris suum* according to the method of Strejan and Campbell (18), were supplied by Dr. Kurt Bloch, Massachusetts General Hospital, Boston, Mass. 2,4-dinitrophenyl (DNP)-protein conjugates were prepared by the method of Eisen et al. (19, 20), at the following substitution ratios: DNP₉-KLH, DNP₂-ASC, DNP₄₃-BSA. PWM (Sigma Chemical Co.) was used at a final concentration of 0.5 or 1 µg/ml.

Isolation of MNC. Normal volunteers between the ages of 28 and 35 yr with no history of IgE-mediated disease were used as cell donors. Heparinized peripheral blood was drawn by venipuncture and diluted 1:2 with 0.95% physiological saline, then underlayered with Ficoll-Diatrizoate (Litton Bionetics, Inc., Kensington, Md.) at a ratio of 1:2 after a modification of the method of Boyum (21). The tubes were centrifuged for 30 min at 400 g at room temperature, after which the interface cells were collected as mononuclear cells and washed three times in minimum essential medium (MEM).

Culture Method for IgE Biosynthesis. Human MNC were cultured in RPMI-1640 (Irvine Scientific, Santa Ana, Calif.) supplemented with 2.5 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 10% fetal calf serum (FCS) as a culture medium. FCS was screened before use for its ability to support optimal IgE biosynthesis by MNC. A total of 3×10^6 cells in 2 ml of culture medium were dispensed into 12- × 75-mm polystyrene tubes (2054; Falcon Plastics, Div. of Becton, Dickinson & Co., Oxnard, Calif.) with or without PWM and/or stimulating antigens, and cultured in a 5% CO₂ humidified atmosphere at 37°C for 3 d. At the end of the incubation, the cells were washed three times with MEM to remove PWM, stimulating antigens, or cytophilic IgE molecules released from the cells during the cultivation, then resuspended in culture medium and plated on either 96-well V-bottomed microplates (Linbro Scientific, Inc., Hamden, Conn.), 200 µl/well containing 3×10^5 cells, or on 24-well macroplates (Linbro Scientific, Inc.), 2 ml/well containing 3×10^6 cells. These plates were incubated in a 5% CO₂ humidified atmosphere at 37°C for an additional 6 d. At the conclusion of the second cultivation, culture supernatant fluids were harvested by centrifugation and kept at -20°C until assayed.

RIA. Total IgE and IgG protein concentrations in culture supernatant fluids were measured by solid-phase RIA in triplicate on 96-well flexible polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.; 22). Properties of the reagents employed, the specificity, the high level of sensitivity, and the exact procedures followed are described in detail elsewhere.²

Briefly, for measurement of IgE or IgG synthesis, 100 µl of samples to be assayed were incubated for 3 h at room temperature on polyvinyl plates previously coated with goat anti-human IgE (GAHE) or goat anti-human IgG (GAHG) protein. After extensive washing of the plates, 100 µl of affinity-purified GAHE or GAHG, at a concentration of 200 ng/ml and radiolabeled to high specific activity with ¹²⁵I (New England Nuclear, Boston, Mass.), was added to each well and the plates were incubated for 3 h at room temperature. After washing the plates thoroughly, the wells were cut and the radioactivity of individual wells was counted on an automatic gamma center. For each assay, IgE and IgG standard curves were constructed, and the straight portions of the curves were used for measurements of IgE and IgG protein concentrations. It should be noted that in our hands, the RIA for IgE allows detection of IgE protein down to the level of 6-12 pg/ml.

Measurement of DNP-specific human immunoglobulins was made with polyvinyl plates coated with 100 µg/ml of DNP-BSA; the remainder of the assay was as above.

Statistical analyses were made with logarithmically transformed data and comparisons by Student's *t* test.

² Zuraw, B. L., M. Nonaka, C. H. O'Hair, and D. H. Katz. Human antibody synthesis in vitro: stimulation of IgE responses by pokeweed mitogen and selective inhibition of such responses by human suppressive factor of allergy (SFA). Manuscript submitted for publication.

Results and Discussion

At the outset, it should be emphasized that certain aspects of the technical details of the culture system that we have established are critical for obtaining the types of results presented here. In particular, establishing cultures in a two-stage process, the first being a stimulation stage in which cells are exposed to antigen and/or PWM, and the second being a synthesis stage in which cells are cultured in the absence of any additional exposure to such substances, appears to be crucial. A detailed report on the studies that established optimal kinetics, cell densities, doses of PWM, dependence of such responses on T cells, and the requirement for IgE-bearing precursor lymphocytes will be published elsewhere.² In the present studies, those conditions that have generally been found to be optimal have been employed.

Stimulation of Human In Vitro IgE Antibody Synthesis by Soluble Hapten-Protein Conjugates. All of the previously published work on human in vitro IgE antibody synthesis has involved either stimulation of human MNC by polyclonal agents such as PWM (12-16), or with specific antigens to which the donor individuals were known to be sensitized (i.e., secondary in vitro stimulation by antigen; 17). We were interested in ascertaining whether human MNC could be stimulated to synthesize IgE molecules by exposure to a typical hapten-protein conjugate, such as DNP-KLH or DNP-ASC. The results of two such experiments are summarized in Fig. 1. MNC from normal human volunteers were preincubated with varying concentrations of either DNP-ASC (experiment I) or DNP-KLH (experiment II) for 3 d in polystyrene tubes. After this first-stage stimulation culture, the cells were thoroughly washed and then plated in 24-well macrocultures. In the absence of any stimulation, MNC of normal donors synthesized IgE in such cultures in amounts around 100 µg/ml. It is clear that

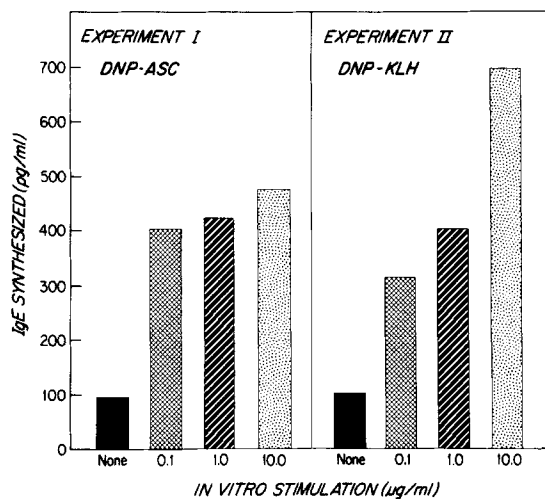


FIG. 1. Stimulation of primary in vitro IgE antibody synthesis by preincubation of human peripheral blood lymphocytes with DNP-protein conjugates. 3×10^6 MNC were cultured in tubes for 3 d either alone or with various doses of DNP-ASC or DNP-KLH. After this first-stage incubation, cells were washed thoroughly and cultured in triplicate macrowells for an additional 6 d. IgE protein values depicted represent the mean of triplicate analysis of each individual culture well.

preincubation with either DNP-ASC or DNP-KLH stimulated significantly enhanced ($P < 0.05$) levels of total IgE protein synthesis.

It should be noted that the ability to stimulate IgE synthesis by exposure to DNP-protein conjugates alone is subject to variability, not only from one individual donor to the next, but also with cells taken from the same donor at different times. In dose-response experiments, optimal enhancement of IgE synthesis by DNP-protein conjugates was found to vary from 0.1 to 50 $\mu\text{g}/\text{ml}$. When a single dose of DNP-protein conjugate was employed for stimulation, ~50% of experiments showed total IgE enhancement of at least 50% over unstimulated cultures. For DNP-protein conjugate plus PWM-stimulated cultures, the percentage of experiments that showed enhanced IgE synthesis compared with stimulation with PWM alone (see below) rose to almost 80%. As detailed elsewhere,² this variability appears to be affected by factors such as total quantities of MNC obtained in a given MNC separation, the temporal relationship of blood collection to occurrences of upper respiratory infections, and to a range of ill-defined elements that can affect the general state of health of normal human beings. To minimize such variability, we have generally found it necessary to use various doses of DNP-protein conjugates each time a new culture is established.

Stimulation of IgE Synthesis In Vitro by PWM or a Mixture of PWM plus DNP-Protein Conjugates. As stated above, it is well known that PWM is effective in stimulating polyclonal in vitro synthesis of immunoglobulins of various classes. In our hands, PWM stimulated increased total IgE synthesis in vitro by human MNC. Thus, as shown in Fig. 2, MNC from two normal volunteer donors responded to exposure to PWM by synthesizing high levels of IgE protein in vitro (cultures III and VII). Moreover, preincubation of the same cells with a mixture containing PWM plus DNP-protein conjugates resulted in even further enhancement of the total IgE protein synthesis. Thus, lymphocytes of both subject A and subject B were stimulated to

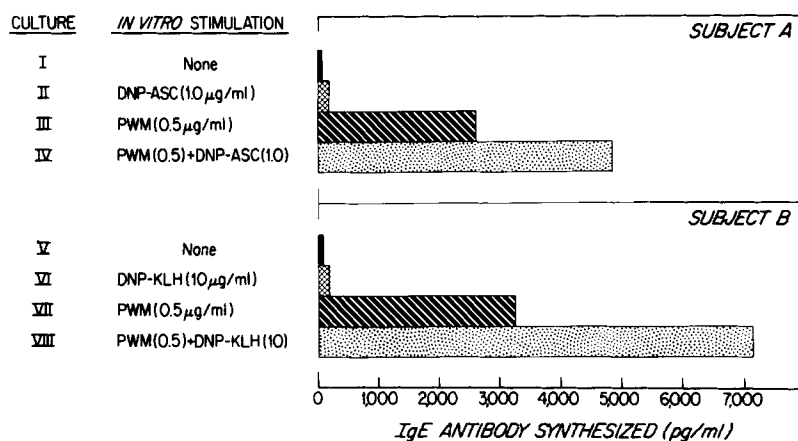


FIG. 2. Stimulation of primary in vitro IgE antibody synthesis by preincubation of human peripheral blood lymphocytes with DNP-protein conjugates either without or together with PWM. 3×10^6 MNC were cultured in tubes for 3 d either alone or with the antigens and/or mitogens indicated, after which cells were washed thoroughly and cultured for an additional 6 d. Cells of subject A (top panel) were cultured in microwells (10 replicates per culture group), whereas cells from subject B (bottom panel) were cultured in triplicate macrowells. IgE protein values represent the mean of triplicate analysis of each culture well (macrocultures) or each pool of 10 wells (microcultures).

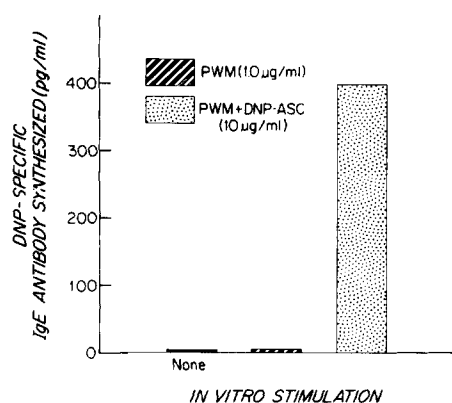


FIG. 3. DNP-specific IgE antibody production in vitro by human peripheral blood lymphocytes stimulated by preincubation with DNP-ASC together with PWM. 3×10^6 MNC were cultured in tubes for 60 h either alone, with PWM, or with a mixture of PWM plus DNP-ASC at the concentrations indicated. At the end of this first-stage incubation, cells were washed thoroughly and cultured in duplicate macrowells for an additional 6 d. Culture supernatant fluids were assayed on DNP-BSA-coated polyvinyl plates and the values depicted represent the mean of triplicate analysis of individual culture supernatant fluids.

synthesize IgE to a significantly greater extent ($P < 0.05$) by the mixture of PWM plus DNP-ASC (culture IV) or PWM plus DNP-KLH (group VIII) than by PWM alone (cf. cultures III and VII) or by either DNP-protein conjugate alone (cultures II and VI).

DNP-specific IgE Antibodies Synthesized In Vitro by Human MNC. Fig. 3 demonstrates the quantities of DNP-specific IgE antibodies synthesized by cultures stimulated by exposure to a mixture of PWM plus DNP-ASC. The RIA for measuring DNP-specific human IgE was set up in a fashion identical to the RIA for total IgE with the exception that the assay plates were coated with DNP-BSA rather than GAHE protein. Because we have no DNP-specific human IgE available, we arbitrarily assigned quantitative values to the DNP binding assay based on the standard curve for measuring total IgE; it should be noted, therefore, that the values presented are not to be construed as absolute values. As shown in Fig. 3, it is quite clear that human MNC either stimulated with PWM alone or not stimulated failed to synthesize detectable levels of DNP-specific IgE; in contrast, MNC exposed to PWM together with DNP-ASC synthesized appreciable levels of anti-DNP antibodies of the IgE class. Although not shown, these same culture supernatant fluids were devoid of DNP-specific antibodies of the IgG class.

Both total and DNP-specific IgE antibodies from three experiments are shown in Table I. Each experiment includes the results from unstimulated, PWM only, and PWM plus DNP-protein conjugate-stimulated cultures. DNP-specific IgE antibodies were detected only in those cultures that were stimulated with PWM plus DNP-protein conjugates. There was no correlation between total IgE levels in the PWM-stimulated cultures and DNP-specific IgE antibodies ($r = 0.35$ by regression analysis).

Table II shows geometric means of total and DNP-specific IgE antibodies from 15 different donors. These results tabulate 51 DNP-specific IgE determinations performed on cultures stimulated with PWM plus DNP-protein conjugates. The only selection criterion employed was that the total IgE from the antigen plus PWM-stimulated

TABLE I
DNP-specific IgE Antibody Production In Vitro by Human MNC Stimulated with PWM Plus DNP-Protein Conjugates

Experiment	IgE*	In vitro stimulation			DNP-specific stimulation index§
		None	PWM	PWM DNP-protein‡	
I	Total	315	808	1,878	10.05
	DNP-specific	0	15	516	
II	Total	145	2,651	10,857	3.82
	DNP-specific	0	8	155	
III	Total	119	2,425	3,578	4.40
	DNP-specific	0	6	170	

* Total IgE (pg/ml) measured by a solid-phase RIA: polyvinyl wells were coated with a normal human serum (NHS)-adsorbed IgG fraction of GAHE and IgE in samples was detected by ¹²⁵I-labeled affinity-purified GAHE. DNP-specific IgE antibodies were similarly measured except that the wells were coated with DNP-BSA. The values presented are specific cpm bound after subtracting background cpm (range, 50–57).

‡ 3×10^6 MNC were cultured in tubes for 3 d with or without stimulation, after which cells were washed and cultured in macrowells or microwells for 6 more d without stimulation. DNP-KLH was used in experiments I and III; DNP-ASC was used in experiment II.

§ The stimulation index was calculated by dividing anti-DNP cpm by background cpm; values less than parity were assigned the value 1.0.

TABLE II
DNP-specific IgE Antibody Production In Vitro by Human MNC Stimulated with PWM Plus DNP-Protein Conjugates*

Culture type	Stimulation	Number assayed	Total IgE pg/ml \pm SEM‡	DNP-specific IgE, stimulation index \pm SEM‡
1	None	15	149 \pm 86	1.10 \pm 0.34
2	PWM only	31	2,003 \pm 681	1.29 \pm 0.32
3	PWM + DNP-protein	51	4,979 \pm 1,251	1.92 \pm 0.49 ($P = 0.001$ vs. 1) ($P = 0.002$ vs. 2)

* 3×10^6 MNC were cultured in tubes for 3 d with or without stimulation, after which cells were washed and cultured in macrowells or microwells for 6 more d without stimulation.

‡ Total IgE (pg/ml) measured by a solid-phase RIA: polyvinyl wells were coated with an NHS-adsorbed IgG fraction of GAHE and IgE in samples was detected by ¹²⁵I-labeled affinity-purified GAHE. DNP-specific IgE antibodies were similarly measured except that the wells were coated with DNP-BSA. The stimulation index was calculated by dividing anti-DNP cpm by background cpm; values less than parity were assigned the value 1.0. All results are geometric means. Because DNP-specific IgE antibodies from PWM, and especially PWM plus antigen-stimulated cultures, were often remeasured in repeat assays, the number of determinations listed for each culture type is different.

cultures was at least 50% above that from the matched cultures stimulated with PWM alone, i.e., that there was antigen-induced enhancement of total IgE synthesis. As shown in Table II, the stimulation index for PWM plus DNP-protein conjugate-stimulated cultures was significantly higher than that seen for PWM only ($P = 0.002$) or unstimulated ($P = 0.001$) cultures.

In contrast to the above results, cultures stimulated with PWM plus DNP-protein conjugate that manifested less than a 50% increase in total IgE synthesis over corresponding controls stimulated with PWM alone displayed a mean DNP-specific

IgE stimulation index of 1.58; this was not significantly different from stimulation with PWM alone (mean = 1.28; data not shown).

The system reported here provides us with a new tool for analyzing various aspects of regulatory control of human antibody responses in general, and of the IgE class in particular. The fact that it is possible to stimulate *in vitro* IgE synthesis by DNP-protein conjugates makes it possible to analyze antigen-specific regulatory mechanisms in the human IgE system paralleling those that have been conducted heretofore in experimental animals. Moreover, this *in vitro* IgE antibody system with human MNC provides us with the necessary tools to examine IgE-selective controlling mechanisms and the manner in which they operate normally in non-atopic individuals, abnormalities that may exist in atopic individuals, and, most importantly, what types of manipulation may be effective in reversing such abnormalities as one approach to solving IgE-mediated allergic diseases of man.

Summary

Experiments are presented herein that demonstrate the capacity to stimulate human peripheral mononuclear cells to synthesize and secrete significant quantities of IgE molecules *in vitro* by exposure to appropriate concentrations of 2,4-dinitrophenyl (DNP)-protein conjugates, pokeweed mitogen (PWM), or a combination of DNP-proteins and PWM. Cultures stimulated in this fashion synthesize increased quantities of both total IgE and DNP-specific IgE antibody molecules. This *in vitro* human IgE antibody system should provide a useful tool for further exploration of regulatory control of IgE responses in both normal humans and those manifesting various forms of IgE-mediated allergic disorders.

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References

1. Ishizaka, K. 1976. Cellular events in the IgE antibody response. *Adv. Immunol.* **23**:1.
2. Katz, D. H. 1978. The allergic phenotype: manifestation of "allergic breakthrough" and imbalance in normal "damping" of IgE antibody production. *Immunol. Rev.* **41**:77.
3. Katz, D. H. 1979. New concepts concerning the clinical control of IgE synthesis. *Clin. Allergy.* **9**:609.
4. Katz, D. H. 1980. Recent studies on the regulation of IgE antibody synthesis in experimental animals and man. *Immunology.* **41**:1.
5. Waldmann, T. A., S. Broder, R. M. Blaese, M. Durm, M. Blackman, and W. Strober. 1974. Role of suppressor T cells in pathogenesis of common variable hypogammaglobulinemia. *Lancet.* **II**:609.
6. Bona, C., S. Broder, A. Dimitriu, and T. A. Waldmann. 1979. Polyclonal activation of human B lymphocytes by nocardia water soluble mitogen (NWSM). *Immunol. Rev.* **45**:69.
7. Fauci, A. S. 1979. Human B cell function in a polyclonally induced plaque forming cell system. Cell triggering and immunoregulation. *Immunol. Rev.* **45**:93.
8. Chiorazzi, N., S. M. Fu, and H. G. Kunkel. 1979. Induction of human antibody responses *in vitro* with emphasis on allogeneic helper factors. *Immunol. Rev.* **45**:219.
9. Dosch, H.-M., and E. W. Gelfand. 1979. Specific *in vitro* IgM responses of human B cells: a complex regulatory network modulated by antigen. *Immunol. Rev.* **45**:243.

10. Geha, R. S. 1979. Regulation of human B cell activation. *Immunol. Rev.* **45**:275.
11. Gleich, G. J., A. K. Averbeck, and H. A. Svedlund. 1971. Measurement of IgE in normal and allergic serum by radioimmunoassay. *J. Lab. Clin. Med.* **77**:690.
12. Buckley, R. H., and G. Becker. 1978. Abnormalities in the regulation of human IgE synthesis. *Immunol. Rev.* **41**:288.
13. Fiser, P. M., and R. H. Buckley. 1979. Human IgE biosynthesis *in vitro*: studies with atopic and normal blood mononuclear cells and subpopulations. *J. Immunol.* **123**:1788.
14. Saxon, A., and R. H. Stevens. 1979. Stimulation and regulation of human IgE production *in vitro* using peripheral blood lymphocytes. *Clin. Immunol. Immunopathol.* **14**:474.
15. Tjio, A. H., W. M. Hull, and G. J. Gleich. 1979. Production of human immunoglobulin E antibody *in vitro*. *J. Immunol.* **122**:2131.
16. Pryjma, J., J. Munoz, G. Virella, and H. H. Fudenberg. 1980. Evaluation of IgM, IgG, IgA, IgD, and IgE secretion by human peripheral blood lymphocytes in cultures stimulated with pokeweed mitogen and *Staphylococcus aureus* Cowan 1. *Cell. Immunol.* **50**:115.
17. Urena, V., R. Garcia, C. Vela, F. Ortiz, and C. Lahoz. 1979. *In vitro* production of IgE and IgG by sensitized human peripheral lymphocytes. *Int. Arch. Allergy Appl. Immunol.* **59**:135.
18. Strejan, G., and D. H. Campbell. 1967. Hypersensitivity of ascaris antigens. I. Skin-sensitizing activity of serum fractions from guinea pigs sensitized to crude extracts. *J. Immunol.* **98**:893.
19. Eisen, H. N., M. E. Casten, and S. Belman. 1954. Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitation reaction. *J. Immunol.* **73**:296.
20. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1970. Carrier function in anti-hapten immune responses. I. Enhancement of primary and secondary anti-hapten antibody responses by carrier preimmunization. *J. Exp. Med.* **132**:261.
21. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97):77.
22. Zollinger, W. D., J. M. Dalrymple, and M. S. Artenstein. 1976. Analysis of parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens. *J. Immunol.* **117**:1788.