the serum enzymes (*6*), its effect on the tissue enzymes was examined in parallel experiments in which L-methionine was added to the substrate solution to yield a final concentration of 0.01 mole per liter.

The electrophoretic mobilities of the tissue enzymes were compared with the mobility of the normal serum enzyme (Fig. 1). An enzyme identical in electrophoretic behavior to the serum enzyme was present in almost all tissues. The ubiquitous presence of this band may have resulted from contamination by blood since the tissues were not perfused prior to extraction. This may not be the case with liver, however, since a particle-bound enzyme similar to that in serum has been isolated from this organ (*2*).

In addition to the serum-like enzyme, the electrophoregrams of several tissues showed other distinct components of enzymatic activity with mobilities greater or less than that of the serum enzyme.

Extracts of normal and cancer tissue specimens of the same organ (liver, breast, lymph node) exhibited similar electrophoretic patterns. However, the enzymatic activity in the bands of cancer tissue extracts were generally higher than those of the normal tissue.

The electrophoretic patterns of tissue enzymes incubated with substrate in the presence of L-methionine indicated definite inhibition in all cases of the enzyme band corresponding to serum naphthylamidase. However, the tissue components which differed from the serum enzyme were either inhibited, unaffected, or activated by this amino acid. These differences in response to L-methionine were also demonstrated when 1-ml portions of tissue extracts were assayed for their total naphthylamidase activity in the presence and absence of this amino acid. L-Methionine (0.01 mole/liter) yielded 60 percent inhibition of the normal serum enzyme. The effect on tissue enzymes expressed as percentage of inhibition varied as follows: pancreas, 62; kidney, 58; jejunum, 53; colon, 52; liver, 47; breast, 32; urinary bladder, 29; placenta, 24; lymph node, 19; and spleen, 15. In addition, the total naphthylamidase activity of skeletal (diaphragmatic and rectus) and cardiac muscle was increased rather than decreased by L-methionine. The electrophoregram of the muscle extracts showed that it was the fast band of naphthylamidase activity which was activated (Fig. 2).

These results indicate the heterogeneity of tissue enzymes which catalyze the hydrolysis of L-leucyl-β-naphthylamide. These data do not permit the absolute classification of these enzymes either as isoenzymes or totally different molecular species, since their association with other proteins in different tissues may contribute significantly to observed dissimilarities in electrophoretic mobility. In any event, the varied response of the tissue amino acid naphthylamidases to L-methionine serves to establish the existence of biochemical differences among these enzymes.

Chromatographically distinct forms of amino acid naphthylamidase have been demonstrated recently in human liver, small intestine, and pancreas (*7*).

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1. This enzyme was originally considered to be identical with "leucine aminopeptidase" [E.C.-3.4.1.1. in *Enzyme Nomenclature,* (International Union of Biochemistry, Elsevier, New York, 1965)], which catalyzes the hydrolysis of leucylglycine and leucineamide. However, other studies (*2*) have shown that the enzyme is neither identical with aminopeptidase nor specific for leucyl napthylamide. The enzyme has not been given an International Union of Biochemistry code number. However, according to I.U.B. recommendations, its systematic name would be "aminoacylnapthylamide amidohydrolase." In this report we have chosen to use the trivial name "amino acid napthylamidase" (or simply "napthylamidase") in referring to the enzyme.
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Natural Human Antibodies to Gram-Negative Bacteria: Immunoglobulins G, A, and M

Abstract. *Significant amounts of immunoglobulins G and M, and a small amount of natural antibodies reactive with* Neisseria gonorrhoeae *and* Escherichia coli, *have been detected in human adult serums by immunofluorescent techniques. Umbilical cord serums also contained substantial immunoglobulin-G antibody but little or no M or A. These findings challenge the concept that natural antibodies reactive with Gram-negative bacteria are primarily of the immunoglobulin-M class.*

In this report we present evidence that natural human antibodies reactive with Gram-negative bacteria are not limited to the IgM class, but are present in all three major classes of immunoglobulins: IgG, IgA, and IgM. In general, we used fluorescein-conjugated antiserums specific for IgG, IgA, and IgM in an indirect type of fluorescent antibody procedure.

Natural antibodies may be defined as those not related to obvious immunization or specific infection (*1*). Natural antibodies to Gram-negative bacteria are widespread among mammalian species and have long been the subject of investigation and controversy regarding their relation to antibodies associated with immunization or infection (*2*). Studies (*2*) based on a sensitive bactericidal test have suggested that natural and immune antibodies differ in physicochemical properties. Other studies (*3–5*) have resulted in the conclusion that natural human antibodies to Gram-negative bacteria are principally of the γ-macroglobulin (IgM) class (19*S*), and it has been suggested (*3*) that differences between natural and immune antibody result from the properties of two different classes of immunoglobulin (IgG and IgM). However, we have demonstrated in human serums significant natural IgG as well as IgM (and some IgA) antibodies that react with Gram-negative bacteria (*Neisseria gonorrhoeae* and *Escherichia coli*).

We used the following fluorescent-antibody method: A uniform concentration of bacteria (*6*), treated with Formalin (3 percent) or heat (100° or 121°C), was fixed to a circumscribed area of a glass slide. One drop of the

test serum or fraction was placed over the area and incubated for 30 minutes at 37°C. The slide was washed for three 5-minute periods in buffered saline (*p*H 7.2) and dried. One drop of the appropriate fluorescent antibody to globulin (*7*) was then placed over the bacterium-antibody complexes, and the slide was incubated and washed as before. The slides were examined with a fluorescence, darkfield microscope (*8*). Fluorescence scored as 2+ or greater was considered a positive indication of antibodies of the specific immunoglobulin class. Appropriate controls were included in each test.

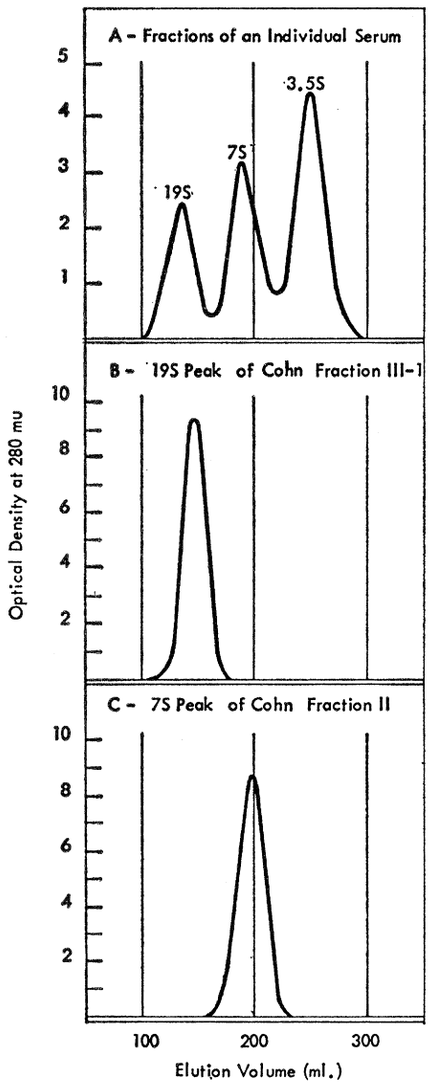

Fig. 1. (*A*) Gel filtration on Sephadex G-200 of the serum of a normal adult. Typical separation into 19*S*, 7*S*, and 3.5*S* peaks (*11*). (*B*) The 19*S* peak from Cohn fraction III-1. Within the pooled elution volumes comprising this peak the fluorescein-conjugated antiserum to IgM detected IgM antibody activity to a titer of approximately 1 : 400 to *Escherichia coli* and 1 : 200 to *Neisseria gonorrhoeae*. (*C*) The 7*S* peak from Cohn fraction II. The fluorescein-conjugated antiserum to IgG detected IgG antibody activity to a titer of approximately 1 : 600 to *E. coli* and 1 : 300 to *N. gonorrhoeae*. IgM activity was not demonstrable within this peak.

The specificity of the antiserums for IgG, IgA, and IgM in our test system was confirmed (*9*) both by fluorescent-antibody reactivity and gel precipitation with chromatographically and electrophoretically purified serum immunoglobulin fractions, by cross absorption with these fractions, and by gel-diffusion comparison with reference antiserums (*10*).

The natural antibodies from the following human sources were characterized: (i) serums from 20 normal adults, (ii) Cohn fractions II and III-1 of pooled normal serums (*7*), and (iii) pooled umbilical cord serums (*7*).

In each of the 20 adult serums tested we found that the titer of IgG antibodies reactive with heat- or Formalin-treated *E. coli* and *N. gonorrhoeae* was equal to or greater than the titer of IgM antibodies to these antigens; IgA was detectable in lower titers (Table 1).

In order to confirm the class identity of the antibodies demonstrable in whole serums, five individual serums and Cohn fractions II and III-1 were fractionated into 19*S* and 7*S* components (*11*) by gel-filtration on Sephadex G-200 (*12*) (Fig. 1). This physical separation of IgG and IgM also minimized any influence on test results of possible (though undetected) anomalies in the specificities of the fluorescent antibody reagents for detecting immunoglobulin. The 7*S* protein peak of Cohn fraction II and of each of the five serums contained the bulk of the IgG molecules as measured by radial diffusion techniques (*13*) as well as most of the IgG active in the fluorescent-antibody tests. Neither IgM antibody activity nor IgM immunoglobulin could be detected in the material from these 7*S* peaks. The immunoglobulin and the antibody activities in the case of IgM were confined to the 19*S* peak of Cohn fraction III-1 and of each individual serum.

It has been concluded that the human placenta prevents the passive transfer of maternal IgM and IgA to the fetus without hindering the passage of IgG antibodies (*4, 5*). We therefore studied pooled cord serum and found in it IgG reactive with both *E. coli* and *N. gonorrhoeae* at titers equal to that of most adult serums. Little or no IgM or IgA fluorescent-antibody activity was demonstrable (Table 1). These results were confirmed by examining 20 matched pairs of maternal and cord serums. We found that IgG titers were approximately equal in each serum pair, whereas only the maternal serums contained significant titers of IgA and IgM (*9*).

Table 1. Antibodies (IgG, IgM, and IgA) in whole serums of 20 adults and in pooled umbilical cord serum. Parentheses indicate range.

Bacteria*	Reciprocal of serum titer		
	IgG	IgM	IgA
	Adult serum		
GC	64†	16	2
	(16–256)‡	(8–32)	(2–4)
EC	32	16	2
	(16– 64)	(8–32)	(N–4)
	Cord serum		
GC	40	N§	N
EC	40	N	N

* Cultured for 15 hours on agar, heated (121°C for 2 hours) and fixed on glass slide (see text). GC: *N. gonorrhoeae* F62-T1, EC: *E. coli* O86: B7:NM. † Mean value of reciprocal of highest reactive dilutions. ‡ Range of reactive titers. § N: Undiluted serum was not reactive or failed to achieve a fluorescence of 2+.

We have also used immunofluorescent techniques to demonstrate that human complement (*14*) could be fixed by the IgG-bacterium complex as well as by the IgM-bacterium complex (*9*).

Our findings, which suggest that a major portion of natural human antibody to Gram-negative bacteria is of the IgG class, challenge the concept (*1, 3, 4*) that natural human antibodies are principally of the IgM class. It appears that the substantial contribution of the IgG natural antibodies has previously evaded detection. This may be due to the relative inefficiency of IgG in certain tests. For example, rabbit IgM antibody was 22 to 1000 times more efficient than IgG antibody in agglutination, bacteriolytic, and opsonization tests (*15*). Also, the hemolytic and hemagglutinating activities of rabbit IgM have been shown to be 60 to 160 times higher on a molar basis than those of IgG measured in a specific hapten-erythrocyte system (*16*). It seems that human antibody classes may react in a similar manner. We have found that natural human IgG antibody to *N. gonorrhoeae* is relatively inefficient in bactericidal and agglutination reactions (*9*).

In addition the demonstration that

IgM antibody to *Salmonella typhosa* "O" antigen fixes complement so efficiently that "whole antiserum will behave like the 19*S* fraction" (*17*) suggests that generalizations concerning immunoglobulin classes based on the results of complement-dependent bactericidal tests should be made with caution.

The demonstration of natural antibodies to *E. coli* and *N. gonorrhoeae* in cord serums has bearing on the study of resistance to infection in newborns. The relative susceptibility of newborns to infection with Gram-negative bacteria might be explained by their deficiency of natural IgM bactericidal antibody (*4*). However, several observations suggest that IgM bactericidal activity is not the principal means of defense. Although virtually all newborn infants lack IgM bactericidins to an equal degree and all are exposed to a potentially pathogenic flora at birth, only a very small number succumb to infection with Gram-negative bacteria (*1*, *5*). The finding that the cord serums examined contained natural antibodies of the IgG class suggests that such IgG antibodies may contribute to the resistance demonstrated by most newborns to Gram-negative bacterial infections. Although IgG antibody may appear to be less reactive in bactericidal tests, it may perform a critical function within the host's antibacterial recognition system.

The current concept (*18*) that a sequential appearance of IgM and IgG antibodies occurs after immunization should be reexamined in the light of our finding that natural IgG antibody in humans may be present prior to immunization or symptomatic infection. The concept of a sequential immunoglobulin response has also been questioned in a study of antibodies produced by immunized rabbits (*19*).

In summary, immunofluorescence techniques can reveal important antibody activity which may elude detection by other methods. It appears that the natural humoral recognition systems for the heat-stable somatic antigens of Gram-negative bacteria are not limited to IgM antibodies alone and that a major portion of this natural antibody activity resides in the IgG class.

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Immunoglobulin Synthesis in vitro by Established Human Cell Lines

Abstract. *Several lines of human lymphoid cells derived from malignant lymphoma produce immunoglobulins in vitro. Immunoglobulins G(IgG, γG) are synthesized in two cell lines and immunoglobulin M (IgM, γM) in one.*

Continuous production in vitro of antibody or other immunoglobulins by human cells has not been reported. Until 1964, no continuous cultures of human lymphoid or plasma cells were available. Pulvertaft and Epstein and his associates first described propagation in vitro of lymphoid cells in suspension culture from malignant lymphomas of African children (*1*) and, subsequently, similar cell lines have been isolated in several other laboratories (*2*, *3*). The patients from whom these cells were derived had maglignant lymphomas with clinical and pathological features which Burkitt and O'Conor described in most cases of childhood lymphoma in Africa (*4*). These neoplasms are now referred to as "Burkitt's tumors." Dorfman and O'Conor *et al.* (*5*) have described similar tumors in American patients and from one of these, a young American woman, a cell line similar to the lines obtained from the African children has been isolated (*6*). The present studies were carried out with the cell line derived from this American patient, designated AL-2, and with three cell lines from African patients designated AL-1, EB-2, and Ogun (*7*). These lines were maintained in culture in a medium composed of 80 percent mixture No. 199 and 20 percent fetal bovine serum. After 48 hours of cell growth, the culture fluid freed from cells was concentrated 20-fold and examined by immunoelectrophoresis for the purpose of detecting any human immunoglobulins that might be present.

Human immunoglobulins were identified with antiserums specific for IgG, IgA, IgM, or IgD [that is, for polypeptide γ-, α-, μ-, and δ-chains (heavy) of human immunoglobulins] and for molecules with κ and λ light polypeptide chains (*8*). Antiserums were absorbed with fetal bovine serum and with bovine γ-globulin to remove cross-reacting antibodies that might impair detection of human immunoglobulin in the presence of the fetal bovine serum of the culture medium.

Immunoelectrophoresis of the concentrated incubation medium from line AL-2 indicates a relatively homogeneous immunoglobulin in the cathodal (slow gamma) region (Fig. 1). No immunoglobulin was detected in the medium from AL-1 cells. Biosynthetic techniques confirmed the synthesis of immunoglobulin by AL-2 cells and the absence of immunoglobulin formation in the AL-1 line.

The method for detection of im-