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Immunodiagnosis
Antibodies in Body Fluids

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1. Introduction to Antibody Structure

1.1. Antibody Response

The immune system consists of two major components, cellular immunity and humoral immunity. The latter is expressed through the synthesis of soluble antibodies. Under normal conditions, antibodies are formed by bone-marrow-derived lymphocytes (B cells) in reaction to the stimulation of antigens which enter the body by inhalation, ingestion, and/or penetration of the skin. In the laboratory, antibodies are usually produced by deliberate immunization by injection of antigens intravenously, intradermally, subcutaneously, etc., in the presence of adjuvants.

When antigen is introduced into an animal the first time, a primary response is evoked in which antibodies toward the immunogen are detectable after a lag period of 1–30 days. This period varies with dose, route of injection, size and solubility of antigen, type of adjuvant used, and many other factors. The time required to achieve maximum antibody titers and the duration of the peak level also vary with the nature of the antigens and the methods of immunization. For a large antigen (e.g., the red blood cell) the peak is often reached approximately 5 days after injection, whereas for a smaller soluble antigen (e.g., plasma proteins) 10 days or more is required. After the antibody levels in the primary response have declined, a subsequent encounter with the same antigen usually evokes a much stronger secondary response. The secondary response requires a lower threshold dose of immunogen and has a shorter lag period, a faster rate of increase, and a longer persistence of antibody titer at the peak than the primary response. The ability of an individual to mount a secondary response can persist for many years, long after antibodies are no longer detectable in the body. Thus secondary response provides long-lasting immunity against certain types of infections (reviewed in Fudenberg *et al.*, 1978; Roitt, 1974; Eisen, 1974).

During the course of a primary immunization, the antibodies produced at the early stage are predominantly IgM class; in most cases IgM antibodies are gradually replaced by IgG antibodies after a few days. The early IgM and the late IgG antibodies often share idiotypic determinants (Oudin and Michel, 1969). (Idiotypic determinants are antigenic determinants in a population of antibody molecules that are unique to that population and often are not observed in other antibody molecules in the same individual or in antibodies directed toward the same antigen in other members of the same species. Presumably an idiotypic determinant is unique to antibodies produced by a single clone of plasma cells.) There is evidence that some of the IgM-producing cells may become IgG producers during the matur-

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ation of B lymphocytes (Nossal *et al.*, 1973; Greaves, 1971; Pernis *et al.*, 1971); this IgM → IgG switch may involve simultaneous repression of a constant-region gene for a μ chain and derepression of a constant-region gene for a γ chain (Wang *et al.*, 1977). Coexistence of other antibody classes has also been described (Seon *et al.*, 1973; Wolfenstein-Todel *et al.*, 1974; Fair *et al.*, 1975). Studies with chickens indicated that the "switch" takes place in the bursa of Fabricus and follows the sequence IgM→IgG→IgA (Cooper *et al.*, 1972). However, an alternative model was proposed by Warner (1974) and Manning (1974), suggesting that IgM precursor cells may give rise to cells producing other classes of antibodies by separate pathways.

1.2. Structure of Antibodies

Antibodies are highly heterogeneous. Phylogenetic studies indicate that all vertebrates have antibodies, and many of the major breakthroughs in the understanding of antibody structure were made via studies with animals. However, a detailed description of antibody structure in all species is beyond the scope of this chapter, so we shall concentrate our discussion on human antibodies as much as possible.

Five classes of antibodies, termed IgG, IgA, IgM, IgD, and IgE, have been described in humans. Some of these classes are further divided into subclasses. Table 1 shows the physicochemical and biological properties of these molecules. For example, when in aggregated form or combined with antigen, IgM and most of the IgG fix complement through the classical pathway (Muller-Eberhard, 1968). Among the IgG subclasses, IgG3 is most efficient, followed (in decreasing order) by IgG1 and IgG2. The activation occurs via binding of the first complement component (C1) to a site located in the Fc fragment of the IgM or IgG molecule. Other antibodies do not fix complement through the classical pathway; IgA and IgE are capable of activating the complement system through an alternative pathway that involves C3, properdin, factor B, and factor D (Gotze and Muller-Eberhard, 1976; Boackle *et al.*, 1974). It is also of considerable interest that only IgG possesses the ability to cross the placental barrier, whereas all other classes of antibodies do not (Fudenberg *et al.*, 1978). IgG molecules

TABLE 1. Properties of Human Antibodies

	IgG	IgA	IgM	IgD	IgE
H-chain class	γ	α	μ	δ	ε
H-chain subclass	γ1, γ2, γ3, γ4	α1, α2	μ1, μ2		
L-chain type	κ and λ	κ and λ	κ and λ	κ and λ	κ and λ
Molecular formula	γ_2L_2	α_2L_2[a] or $(\alpha_2L_2)_2SC^bJ$	$(\mu_2L_2)_5J^c$	δ_2L_2	ϵ_2L_2
Sedimentation coefficient (S)	6–7	7[a]	19	7–8	8
Molecular weight (approximate)	150,000	160,000[a] 400,000[d]	900,000	180,000	190,000
Electrophoretic mobility (average)	γ	Fast γ to β	Fast γ to β	Fast γ	Fast γ
Complement fixation (classical pathway)	+ + +	0	+ + + +	0	0
Valence	2		10		
Serum concentration (mean mg/ml)	10	2	1.2	0.03	0.0005
Half-life (days)	23[e]	6	5	3	2.5
Placental transfer	+	0	0	0	0
Reaginic activity	?	0	0	0	+ + + +
Antibacterial lysis	+	+	+ + +	?	?
Viral inhibition	+	+ + +	+	?	?

[a] For monomeric serum IgA.
[b] Secretory component.
[c] J chain.
[d] For secretory IgA.
[e] Not including IgG3.

transferred from the mother provide partial protection against infections for newborn babies when their own humoral immune systems have not fully developed.

All antibodies have one or more basic unit(s) consisting of identical pairs of heavy (H) and light (L) polypeptide chains (Fig. 1). [Readers are urged to refer to recent review articles for detailed information regarding antibody structure (Gally, 1973; Wang and Fudenberg, 1974; Frangione, 1975; Capra and Kehoe, 1975).] Under normal circumstances, IgG, IgD, and IgE consist of a single basic unit, IgM of 5 units, and IgA of 1–4 units. Each antibody polypeptide chain has a variable (V) and a constant (C) region. The V region is the amino-terminal portion of the chain, which is made up of many different amino acid sequences. The C region is the carboxyl-terminal portion of the chain, the amino acid sequence of which is the same within a given subclass of H chain and a given subtype of L chain, with the exception of allotypic differences. The V region is approximately 110–120 amino acid residues in length, as is the C region of L chains. However, different classes of H chains differ in their C regions. C_γ and C_α are approximately 3 times longer, whereas C_μ and C_ϵ are 4 times longer than the V regions (Putnam, 1974; Bennich and von Bahr-Lindstrom, 1974).

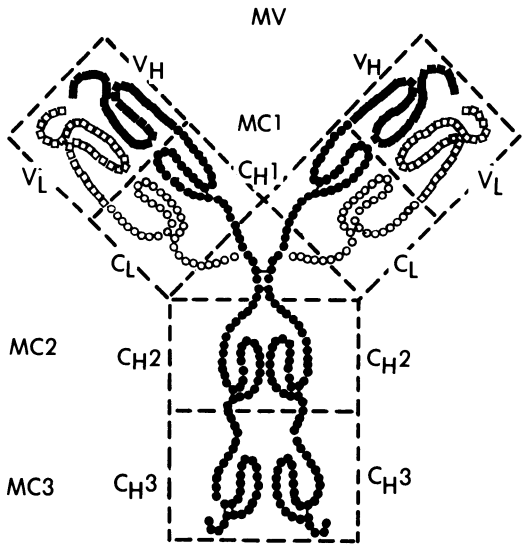

Figure 1. The four-chain basic unit of antibodies. H chains are represented by solid beads and squares, L chains by empty beads and squares. V regions are squares, and C regions are beads. Each dotted large square encloses a module. There are two V-region modules (MV), two C1, one C2, and one C3 module in IgA and IgG. Each module consists of two domains (see text for detailed description).

Each antibody polypeptide chain consists of repeating homology regions, and each homology region is similar in size to a V region and is characterized by an intrachain disulfide bond bridging two cysteine residues approximately 60 residues apart (Pink *et al.*, 1971). Based on the amino acid sequence data, Edelman (1970) hypothesized that each homology region would fold into a somewhat similar configuration, a compact globular domain. Each domain might have evolved to perform a particular biological function. This concept was subsequently confirmed by X-ray crystallographic analyses (Segal *et al.*, 1974; Edmundson *et al.*, 1974; Poljak, 1975). Figure 2 shows the α-carbon backbone of the F(ab) fragment of a human myeloma protein (New) at 2.8 Å resolution. The data show not only that each homology region is folded into a compact domain, but also that the amino acid residues of one domain interact tightly with those of the corresponding domain of the other chain in the same molecule to form a larger globular unit termed a "module" (Nisonoff *et al.*, 1975). On the other hand, the areas between neighboring domains are extended, making them more vulnerable to cleavage by proteolytic enzymes or chemicals. When the intact antibody molecule is subjected to limited enzymatic or chemical treatment, fragments containing discrete domains of various sizes are often obtained. Table 2 summarizes various reports of such fragments described in the literature. Studies with these fragments have contributed greatly to the understanding of the structure–function relationship of antibodies. Through such studies, it is firmly established that the V-region module is responsible for binding to antigen (Eisen, 1971; Singer *et al.*, 1971), whereas C-region modules are responsible for the other biological functions. In man, IgG1, IgG2, IgG3, and IgM fix complement via the classical pathway (Muller-Eberhard, 1968), and the primary binding site of IgG1 molecule to Clq is located at the module containing a pair of C_H2 domains (Ellerson *et al.*, 1972). Similarly, human IgG1 and IgG3 antibodies are cytophilic (Abramson *et al.*, 1970; Huber *et al.*, 1971), and the primary effector site of IgG1 molecules for binding to the membrane of

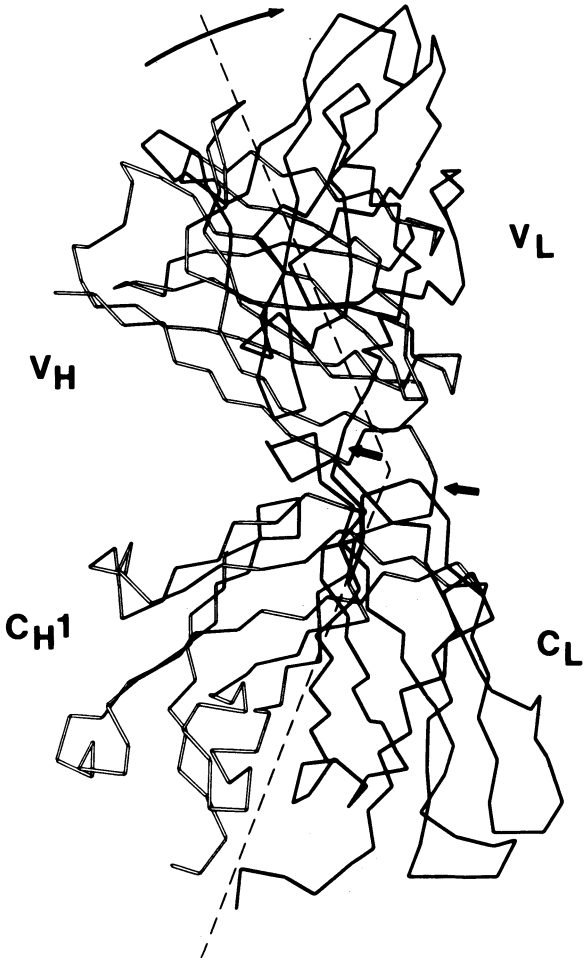

Figure 2. The α-carbon backbones of L (solid line) and H (empty line) chains of the fragment of a human myeloma protein New. The three-dimensional structure was reconstructed based on X-ray crystallography data. Reproduced from Poljak *et al.* (1974) with permission.

monocyte is located on the module containing a pair of C_H3 domains (Yasmeen *et al.*, 1973; Ciccimara *et al.*, 1975). On the other hand, human reaginic antibodies belong to the IgE class (Ishizaka *et al.*, 1966), but the attachment of an IgE molecule to the membrane of a mast cell may involve at least two sites, one at the C_H3 and the other at the C_H4 domain (Bennich and von Bahr-Lindstrom, 1974).

In normal human adults, IgG constitutes approximately 75% of the total serum immunoglobulin. The IgG class is further divided into four subclasses, designated IgG1 through IgG4; the relative concentrations of the four subclasses are approximately 60–70% for IgG1, 14–20% for IgG2, 4–8% for IgG3, and 2–6% for IgG4. IgM constitutes approximately 10% of the total serum antibody. IgG and IgM are mainly responsible for the classical precipitation and agglutination types of antigen-antibody reactions (Heidelberger, 1956). However, in mucosal body secretions, IgA is the predominant antibody class (Tomasi and Grey, 1972; Lamm, 1976). Table 3 shows the approximate concentrations of IgA and IgM in various body secretions. Other classes of antibodies are present in minute amounts in body secretions, but reliable quantitation has not been defined. IgE-producing plasma cells are more frequently found in lymphoid tissue around secretory glands than in lymph nodes and spleen, suggesting that IgE may also be more concentrated in certain body fluids than in serum (Ishizaka, 1970). In body secretions, IgA exists essentially as a dimer of two four-chain basic units stabilized by combination with two other proteins—the secretory component and the J chain (Heremans, 1974; Koshland, 1975). The complex, termed "secretory IgA," has a molecular weight of approximately 400,000. The binding of secretory component to IgA is via noncovalent interactions (Hong *et al.*, 1966), whereas the binding of J chain to IgA is by disulfide bonds which link the penultimate cysteine residue at the *C*-terminal end of the α chain to two of the cysteine residues in the J chain (Mestecky *et al.*, 1974). Within the secretory IgA molecule, no covalent linkage has been found between the secretory component and the J chain.

Based on antigenic differences at the C regions of H chains, IgA molecules are classified into two subclasses, designated IgA1 and IgA2 (reviewed in Heremans, 1974). The amino acid sequence of the C region of the α_1 chain differs from that of α_2 chains at many positions, including an extra 13 amino acid residues at the hinge region (Frangione and Wolfenstein-Todel, 1972). No genetic marker has been discovered for the IgA1 subclass, but two antithetical alleles termed *$A_2m(1)$* and *$A_2m(2)$* have been described for the IgA2 subclass (Kunkel *et al.*, 1969; Vyas and Fudenberg, 1969; van Loghem *et al.*, 1973). Chemical studies indicate that *$A_2m(1)$* molecules lack the inter-heavy-light-chain disulfide bond characteristic of all other antibodies, including those of the *$A_2m(2)$* allotype within the IgA2 subclass (Grey *et al.*,

TABLE 2. Fragments of Antibodies Produced by Limited Enzymatic or Chemical Cleavage

Fragment	Prepared from	Module	Domain	Reference[a]
Fab	IgG	M_V, M_C1	V_L, V_H, C_L, C_H1	1
Fc	IgG	M_C2, M_C3	$(C_H2, C_H3)_2$	1
$F(ab')_2$	IgG	$(M_V, M_C1)_2$	$(V_L, V_H, C_L, C_H1)_2$	2
$F(ab')_2$	IgA	$(M_V, M_C1)_2$	$(V_L, V_H, C_L, C_H1)_2$	3,4
Fc	IgA	M_C2, M_C3	$(C_H2, C_H3)2$	3,4
Fab	IgM	M_V, M_C1	V_L, V_H, C_L, C_H1	5,6
$F(c)_5$	IgM	$(M_C2, M_C3, M_C4)_5$	$(C_H2, C_H3, C_H4)_{10}$	5,6
Fv	IgA	M_V	V_H, V_L	7
Fv	B–J	M_V	V_L or $(V_L)_2$	8–10
$F(ab'')_2$	IgG	$(M_V, M_C1)_2$	$(V_L, V_H, C_L, C_H1)_2$	11
Facb	IgG	$(M_V, M_C1)_2M_C2$	$(V_L, V_H, C_L, C_H1)_2C_H2$	12
Fb(s)	IgG	M_C1	C_L, C_H1	13
pFc′	IgG	M_C3	$(C_H3)_2$	14
stFc	IgG	M_C3	$(C_H3)_2$	15
Fd	IgG		V_H, C_H1	16
Fch	IgG	M_C2, M_C3	$(C_H2, C_H3)_2$	17
[b]	IgG		C_H^2	18–20
[b]	B–J		C_L	8–10
[b]	IgG		V_H	21

[a] References: 1, Porter (1959); 2, Nisonoff *et al.* (1960); 3, Eisen *et al.* (1968); 4, Calvanico and Tomasi (1976); 5, Mihaesco and Seligmann, (1968); 6, Plaut and Tomasi (1970); 7, Inbar *et al.* (1972); 8, Solomon and McLaughlin (1969); 9, Seon *et al.* (1972); 10, Karlsson *et al.* (1969); 11, Cahnmann *et al.* (1966); 12, Connell and Porter (1971); 13, Gall and D'Eustachio (1972); 14, Turner and Bennich (1968); 15, Utsumi (1969); 16, Bjork and Tanford (1971); 17, Michaelsen and Natvig (1973); 18, Ķehoe and Fongerean (1969); 19, Ellerson *et al.* (1972); 20, Seon and Pressman (1975); 21, Dammaco *et al.* (1972).

[b] No name assigned to this fragment.

1968; van Loghem *et al.*, 1973); instead, the two L chains are disulfide-bonded to each other via the *C*-terminal (in κ chains) or penultimate (in α chains) cysteine residue.

1.3. Genetic Control of Antibody Formation

One of the most important breakthroughs in immunogenetics has been the recognition that, in contrast to the one gene–one polypeptide chain rule in molecular biology, at least two genes participate in the synthesis of each antibody polypeptide chain. One gene codes for the V region and another for the C region of each chain (reviewed by Wang, 1978). The V regions have been classified into V_H, V_κ, and V_λ groups according to the degree of amino acid sequence homology, and each group is further divided into several subgroups (Capra and Kehoe, 1975). The V region obtained its name by excessive variability and is responsible for binding to antigens. The number of genes coding for the V regions has been the center of a controversy concerning the genetic control of antibody diversity (reviewed in a book edited by Cunningham, 1976). Two major kinds of hypotheses, namely the somatic and the germ line, have been postulated. The somatic hypothesis suggests that there are only a few V-region genes inherited in the genome, and the diversification of these genes by means of random mutation or recombination in so-

TABLE 3. Concentrations (mg/ml) of IgA and IgM in Body Fluids

	IgA	IgM
Serum	2.0	1.2
Colostrum	12.3	0.6
Jejunal fluid	0.3	—
Colonic fluid	0.8	—
Hepatic bile	0.1	—
Nasal fluid	0.3	—

matic cells is mainly responsible for the generation of antibody variability. In contrast, the germ-line hypothesis proposes that there are a pair of V_H and V_L genes for the synthesis of each antibody molecule and that thousands of V-region genes must be inherited through the germ cells (reviewed by Pink *et al.*, 1971). Neither of these hypotheses can explain all the experimental observations because DNA/RNA hybridization experiments (Honjo *et al.*, 1974; Tonegawa, 1976) indicate that the total number of V-region genes in a mouse is not large enough to generate sufficient variability without somatic diversification. On the other hand, the inheritance of idiotypic determinants in mice (summarized by Weigert and Potter, 1977) and in rabbits (Yarmush *et al.*, 1977) and the sharing of identical V regions by a μ chain and a γ_2 chain in humans (Wang, 1977) suggest that information for producing a specific antibody molecule is stored in the genome. Two additional hypotheses have been advanced recently. One of them suggests that each V region is coded for by several minigenes (or episomes) and that each hypervariable region (and the framework portion) is encoded by a minigene (Wu and Kabat, 1970; Capra and Kindt, 1975). The other hypothesis was proposed by Klinmann and his colleagues (see Metcalf *et al.*, 1977), who believe that predetermined permutation of a moderate number (hundreds) of germ-line genes is responsible for the generation of antibody variability. This model is based on the observation that a smaller (approximately 10^4) but consistent B-cell specificity repertoire exists in young mice, and this repertoire expanded to include more than 10^7 clonotypes in adult mice with or without the influence of antigens. These new hypotheses, together with the allelic exclusion and specific gene activation (Fudenberg *et al.*, 1978), point to the importance of regulatory control of antibody production.

Antibodies are produced by plasma cells. In order for plasma cells to produce a particular antibody, however, complex events requiring interaction of at least two other types of lymphocytes, namely thymus-derived cells (T cells) and macrophages, often occur initially (reviewed by Claman and Mosier, 1972). Genetic studies demonstrated that the ability of an animal to make high antibody titers to many antigens is determined by a set of autosomal dominant "immune response" (*Ir*) genes. To date, *Ir* genes have been identified in mouse (McDevitt and Tyan, 1968), guinea pig (Ellman *et al.*, 1970), rat (Gunther *et al.*, 1972; Armerding *et al.*, 1974), and rhesus monkey (Balner *et al.*, 1973). In all these species, the *Ir* genes are closely linked with genes controlling the major histocompatibility specificities but not with structural genes coding for antibody polypeptide chains (McDevitt *et al.*, 1972; Shreffler and David, 1975). Figure 3 shows the genetic map of the major histocompatibility gene complex of the mouse. At least seven loci were clearly identified, designated *H-2K, Ir-1A, Ir-1B, Ia-3, Ss, H-2G,* and *H-2D.* The entire complex is approximately 0.5 centimorgan in length (equivalent to a 0.5% recombination frequency) and thus contains enough DNA for several hundred genes. The *H-2* complex is arbitrarily divided into five regions and three subregions corresponding to the relative positions of the seven defined loci (Fig. 3). *H-2K* and *H-2D* control the serologically detected *H-2* alloantigens, *H-2G* controls erythrocyte alloantigens, and *Ss* controls serum protein variants. These genes play important roles in tissue transplantation. The two *Ir* genes, termed *Ir-1A* and *Ir-1B,* are located between the *H-2K* and the *Ss* loci. They are identified by immune responses of various inbred strains of mice toward one or more of three types of antigens: (1) synthetic polypeptides with limited structural heterogeneity; (2) alloantigens which differ slightly from their autologous counterparts; and (3) complex multideterminant antigens administered in limiting immunizing doses in conditions under which presumably only the strongest immunogenic determinant(s) are recognized (reviewed by Benacerraf and Katz, 1975).

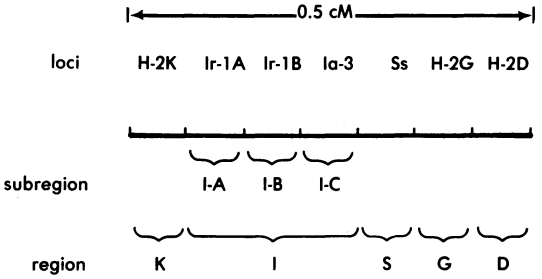

Figure 3. Genetic map of the mouse major histocompatibility complex.

It is noteworthy that only responses to thymus-dependent antigens have been found to be controlled by *Ir* genes; no *Ir* gene has been identified as controlling antibody responses to thymus-independent antigens. It was postulated that *Ir* genes code for a class of molecules produced by antigen-activated T cells which either enhance or suppress the B-cell immune responses, probably by release of soluble products. However, the possible involvement of actual membrane–membrane contact between various immunocytes has not been ruled out. Attempts to detect the products of *Ir* genes by serological methods led to the discovery of a group of lymphocyte alloantigens designated Ia antigens (reviewed by Shreffler and David, 1975). Ia antigens are produced by genes located at the I–C subregion, have a moleular weight ranging from 25,000 to 35,000, and are mainly detected on B cells. Their functional relationship with the *Ir* genes is still not clear.

The effect of *Ir* genes is highly specific. Furthermore, different independent *H-2* haplotypes (a haplotype defines a specific combination of all alleles at all loci within a closely linked genes complex) show different patterns of responses and nonresponses to a panel of antigens. On the basis of these observations, one would expect a large number of *Ir* genes, but, in spite of all-out efforts by many laboratories, only two *Ir* genes have been clearly identified in mice (Lieberman *et al.*, 1972). This serves to remind us that a lot is yet to be learned in this area.

2. Detection of Antibodies in Blood and Cerebrospinal Fluid

2.1. History

In principle, the most satisfactory and direct method for laboratory diagnosis of infection consists of the isolation, cultivation, and/or direct identification of the offending agent. This approach is successful in many instances, such as in the case of some enteric infections, but it is not universally applicable and not always practical, for several different reasons, ranging from the difficulty of growing certain microorganisms in culture to the uncertainty of the causal relation of the cultured agent to the disease.

Since the beginning of the century, many investigators have concentrated on developing methods that would allow the indirect diagnosis of an infection, hoping to find faster and confirmatory techniques for those only involving isolation and/or direct identification of the pathogenic microorganism. The confirmatory use of antibody detection for the diagnosis of infectious diseases cannot be approached in a very rigid way. The knowledge about specific characteristics of the infection to be studied and of the particular clinical problem have to be considered. For example, if the main point is to establish beyond doubt the nature of a given infectious agent that has been responsible for a recent epidemic (as, for example, in cases of viral influenza), one has to rely on the verification of a clear rise in antibody titers; however, if the problem is to establish whether a liver tumor could correspond to liver hydatidosis, the detection of antibodies to *Echinococcus granulosus* extract in one single test is of significance. Similarly, if a patient is suspected of tertiary syphilis, a high antibody titer to *Treponema pallidum* will be confirmatory. Characteristics such as the detection of the immune response, the prevalence of nonpathogenic infestation, cross-reactivity with other microorganisms, etc., are very important in the interpretation of results.

Another important general point to stress is that the detection of antibodies does not necessarily mean that the individual is protected against infection. Lack of protection is almost a rule in fungal and parasitic infections. In viral diseases, the presence of antibodies is an important indicator of future protection mainly against viruses spreading through the blood. However, antibodies will not protect very efficiently against viral infections spreading by fusion of infected cells.

The first technique for specific antibody detection to emerge was that of complement fixation, which by the decade of 1950–1960 had become the backbone of serological diagnosis of infection. However, this is a delicate and time-consuming technique and is not always very effective in early diagnosis, since in several infections complement-fixing antibodies are late in appearance and in many instances

not very long-lasting after their appearance. Furthermore, "infected" sera are often anticomplementary.

The search for other methods based on the demonstration of antibody synthesis vs. any given microorganism has been incessant since midcentury. Agglutination methods, the first alternatives to complement fixation, have been perfected to a considerable level of specificity and sensitivity. Precipitation techniques were offered as a simpler alternative, but only in recent years have they been perfected to a point that their level of sensitivity approaches that of the agglutination procedures. Immunofluorescence techniques have recently been increasing in popularity, due mainly to the introduction of artificial substrates for the antigen–antibody reaction that are susceptible to quantitation by fluorometry, which permit the precise quantitation of antibody without the need for cumbersome titration procedures. Radioimmunoassay and enzymoimmunoassay, the most sensitive techniques presently available, have been recently applied to the diagnosis of infectious diseases with good results.

Two exhaustive reviews of the serological methods most frequently used in diagnosis of human infection have been published: one in the 1975 edition of *Clinical Aspects of Immunology,* edited by Gell, Coombs, and Lachman; the second in the *Manual of Clinical Immunology* edited by Rose and Friedman (1976). In the present text we restrict our attention to recent developments in this field, stressing the lines of progress that we foresee for future approaches to the immunological diagnosis of human infection.

2.2. Antibody Detection in Blood

Blood is the biological fluid most frequently used in diagnostic procedures aimed at the detection of antibodies to suspected microorganisms. Because of the specificity of the methods employed in this detection, screening for large numbers of possible infecting agents is not feasible; therefore, the detection is usually aimed at an infective agent thought to be most likely on clinical and epidemiological grounds. The choice of the method to be used is usually determined by practical availability and by the experience of the person in charge of executing the tests. In general, one or more of five groups of techniques are utilized: complement-fixation tests, agglutination tests, precipitation tests, immunofluorescence tests, and radio- or enzymoimmunoassays. Detection of antibodies by any of these approaches is not proof of active infection. To substantiate such a diagnosis, an increase in antibody titer must be documented under identical assay conditions for two samples collected 7–14 days apart. As in many other areas of laboratory diagnosis, however, one has to be aware of possible causes of error and confusion. It has been known for years that immunizations with pertussis of tetanus antigens may lead to nonspecific increases in antibody titers, for example, to streptococcus and pneumococcus. The reason for this phenomenon seems to lie in the immunostimulatory properties of such antigens. The nature of the immunostimulatory effect is not well understood, but experimental work with T-independent antigens, such as tetanus toxoid or bacterial lipopolisaccharides, this last substance also having immunostimulant properties, have shown that they are able to trigger B cells nonspecifically to produce antibodies against unrelated antigens, behaving as B-cell mitogens (Coutinho and Moller, 1973; Coutinho *et al.,* 1974).

2.2.1. Complement-Fixation Tests

As stated before, complement-fixation (CF) tests have been the backbone of serological diagnosis for most infectious diseases. The classical example is the Wassermann reaction for diagnosis of syphilis, but many other techniques have been described for diagnosis of bacterial, viral, fungal, protozoal, and parasitic infections. The main drawbacks of the technique arise from its relative sophistication and from the fact that it usually detects antibodies of late onset; in several epidemiological studies, CF tests are often unreliable because CF antibodies may fall rapidly after infection with certain agents. Among bacterial diseases, it is rarely applied with success, except in the case of syphilis (Sussman, 1975), but complement-fixation techniques have proved useful in several fungal infections such as coccidiodomycosis and histoplasmosis (Longbottom and Pepys, 1975), in parasitic infections such as trichinosis, echinococcosis, and schisto-

somiasis (Soulsby, 1975), in protozoal infections (Wilson, 1975), and in viral diseases (Nagington, 1975). In this last group of diseases, given their usual acute character, the slow increase in titer of complement-fixing antibody limits the usefulness of the test for diagnostic purposes.

In any case, complement-fixation tests are being gradually replaced by other techniques, and their application probably will become more and more restricted in the future. Unfortunately, many laboratories have become so entrenched with this assay, limited in several ways as to the significance of results obtained, that the transition to better assays will be predictably difficult.

2.2.2. Agglutination Tests

Several types of agglutination procedures can be used diagnostically:

1. Those based on the direct agglutination of a microorganism. A classical example is the Widal reaction for salmonellosis, and similar reactions have been used in the diagnosis of brucellosis, leptospirosis, trypanosomiasis, tularemia, plague, etc. The Weil-Felix reaction for rickettsiosis is similar in concept.
2. Those based on the agglutination or flocculation of inert particles coated with antigen. The classical flocculation tests are the VDRL and Kahn tests for syphilis. The flocculating antigen is a mixture of cardiolipin, cholesterol, and lecithin, which is agglutinated by the antibodies to cardiolipin synthesized by the patients infected with *Treponema pallidum*. Antigen-coated particles of cholesterol or cholesterol-lecithin crystals have also been used in flocculation tests for the diagnosis of helminth infections such as trichinosis and schistosomiasis (Soulsby, 1975). Other types of particles have been used as inert support for flocculation and agglutination tests, including bentonite and latex. Latex agglutination tests have been used in the diagnosis of *Neisseria meningitis* infection, in the screening of antibodies to streptolysis O (Sussman, 1975), in the measurement of antibodies to streptococcal mucopeptides (Heymer *et al.*, 1973), and with variable degrees of success in the diagnosis of fungal diseases such as coccidiodomycosis and histoplasmosis (Longbottom and Pepys, 1975), as well as in some parasitic infections such as echinococcosis (Soulsby, 1975). These techniques are simple and reliable, although sometimes they lack the sensitivity of other classical serological reactions. However, they have been the object of much attention because of their easy standardization and commercialization, allowing the performance of diagnostic tests in the most rudimentary conditions. In some cases, latex agglutination tests have proved superior in sensitivity to other techniques, such as in the case of the test aimed at the detection of antibodies to streptococcal mucopeptides (Heymer *et al.*, 1973).
3. Those based on the inhibition of hemagglutination by viruses. Several viruses can induce the agglutination of selected red cells. For example, vaccinia virus produces two hemagglutinins that will agglutinate only chicken erythrocytes (Anthony *et al.*, 1970). As a result of viral infection, the host produces soluble and structural hemagglutinins that will inhibit the hemagglutinating capacity of the virus. The titration of such HAI antibodies is the main diagnostic technique for rubella and has also been found useful in influenza and arborvirus infections. The main problem of these tests lies in the need to eliminate nonspecific inhibitors, which vary from case to case and usually consist of lipoproteins, mucoproteins, and polysaccharides (Nagington, 1975).
4. Those based on agglutination of antigen-coated erythrocytes. The technique of passive hemagglutination has found a widespread application in diagnostic microbiology. Most polysaccharide antigens will avidly absorb to red cells for use in such tests. Protein antigens can also be coupled to red cells after pretreatment of the cells with a variety of antigens [tannic acid, bis-diazotized benzidine (BDB), glutaraldehyde, chromic chloride, etc.]. The most delicate steps in the technique lie in the pretreatment of red cells. There is evidence suggesting

that chromic chloride is the most simple and reliable reagent to use for such pretreatment (Gold and Fudenberg, 1967; Faulk and Houba, 1973). The sensitivity of passive hemagglutination is usually satisfactory, matching any other conventional serological method. Its main drawbacks are related to the need for specialized technicians and adequate supplies of stabilized sheep red blood cells. This test is not as easy to perform as the agglutination or flocculation technique using latex as an indicator particle. In some bacterial diseases, hemagglutination tests can be very easily developed, since bacterial polysaccharides bind spontaneously to red cells. Such tests have been developed for the diagnosis of plague, *Vibrio fetus,* and *Neisseria meningitis* infections (Sussman, 1975). In urinary tract infections, passive hemagglutination has been indicated as a method potentially capable of detecting those cases with infection of the renal parenchyma (Sussman, 1975). However, in most cases, hemagglutination is used after chemical coupling of antigens to the red cells. Hemagglutination tests based on this principle have been developed for plague, leptospirosis, syphilis (Sussman, 1975), aspergillosis (Tönder and Rodsaether, 1974), amebiasis, trypanosomiasis, toxoplasmosis (Wilson, 1975), and echinococcosis (Soulsby, 1975). In the case of protozoal diseases and in some parasitic diseases, hemagglutination techniques are considered to be the best serodiagnostic techniques available on the basis of their sensitivity and reliability (Wilson, 1975; Soulsby, 1975). The sensitivity of hemagglutination is such that it can be used for the detection of antibodies to commensal bacteria, such as some strains of *Escherichia coli.* The *E. coli* antigens do not require chemical coupling to human group O red blood cells and are easily adsorbed through incubation at 37°C. The detection of such antibodies has been proposed as an indication of the competence of the humoral immune response (Webster *et al.,* 1974).

2.2.3. Precipitation Tests

Precipitation tests have been used mainly in the diagnosis of fungal diseases. Their main advantages are their relative simplicity and reproducibility, but, in general, they have been found to be considerably less sensitive than other currently used serological techniques. In the case of fungal diseases, the sensitivity seems adequate, and the main problems are cross-reactions between fungi and other nonfungal proteins (Longbottom and Pepys, 1975). In any case, precipitation methods have been used for the diagnosis of aspergillosis, candidiasis, and histoplasmosis (Longbottom and Pepys, 1975). In the case of aspergillosis, immunoelectrophoresis has been used as the detection technique, and attempts to graduate the disease according to the number of precipitins have been reported (Longbottom and Pepys, 1964).

In candidiasis, precipitin tests have been used extensively. It seems that two types of antibody can be detected: (1) antibodies directed to capsular polysaccharides, which arise in almost any type of candida infection and can show some cross-reactivity due to the similarities of the capsular polysaccharides in several fungi, and (2) antibodies directed to cytoplasmic antigens, which are more specific but appear only in cases of deep-rooted infection (Longbottom and Pepys, 1975). Standardization of precipitin tests for candida infection has met some problems due to the antigenic complexity of the fungus. As many as 22 different precipitins have been identified by crossed immunoelectrophoresis (Svendsen and Axelsen, 1972), Using double diffusion to screen these precipitins, variable results are obtained according to the type of extract used and the antigen–antibody proportions in the system (Faux *et al.,* 1975). Recently, counter-immunoelectrophoresis (CIE) has been proposed as the most adequate test for routine purposes, because of its greater sensitivity (Odds *et al.,* 1975).

The introduction of CIE has renewed the interest in the application of precipitin tests for the diagnosis of infectious diseases. Classical methods had been used in some instances, e.g., for the detection of antibodies to *Mycoplasma pneumoniae* (Conant *et al.,* 1968), to varicella-zoster virus (Trlifajova *et al.,* 1972),

to *Plasmodium falciparum* (McGregor and Wilson, 1971), and to *Echinococcus granulosus* (Bombardieri *et al.*, 1974). However, the sensitivity of double immunodiffusion can be a serious limitation, and CIE is a simple way of substantially increasing the sensitivity. It is no suprise that applications of this method are being reported with increased frequency. One of the applications of CIE is the titration of antibodies to tetanus and diphtheria toxoids (Sgouris, 1972). These are particularly useful antibodies as indicators of the ability to synthesize antibodies to protein antigens. By titration of the endpoint of the precipitation, a semi-quantitative approach is possible. In our laboratory we have been quantitating these antibodies in young, vaccinated normal children. In 61% of the children studied (a total of 23), we detected antibodies to tetanus toxoid in titers ranging from 1/1 to 1/32 (Fig. 4). The frequency of children with detectable antibodies to diphtheria toxoid was significantly smaller (13%) and the titers lower, ranging from 1/1 to 1/14. Antibodies to vaccinia have been screened by this technique as well, with the aim of selecting plasma suitable for preparing antivaccinia immunoglobulin (Entwistle, 1974).

A common drawback of CIE and hemagglutination techniques is the need to determine end points in serial dilutions to achieve semiquantitation of the antibodies in question. This represents a considerable workload, and the accuracy is never as good as with quantitative techniques. Radial immunodiffusion has been used, for example, to quantitate antibodies to tetanus toxoid (Eldridge and Entwistle, 1975), anti-streptolysin O (Fasth, 1974), and antibodies to streptococcus B in experimental antisera (George and Harrell, 1974). Rocket immunoelectrophoresis has also been used for detection of specific antibodies. Crowle *et al.* (1974) has published an exhaustive assessment of the experimental conditions to be met, and the application of this method to quantitation of tetanus and diphtheria toxoids has been reported (Hoyeraal *et al.*, 1975). With both techniques, the main problem is to obtain adequate sensitivity, and, in the case of rocket immunoelectrophoresis, there are considerable technical difficulties to overcome before satisfactory results are obtained.

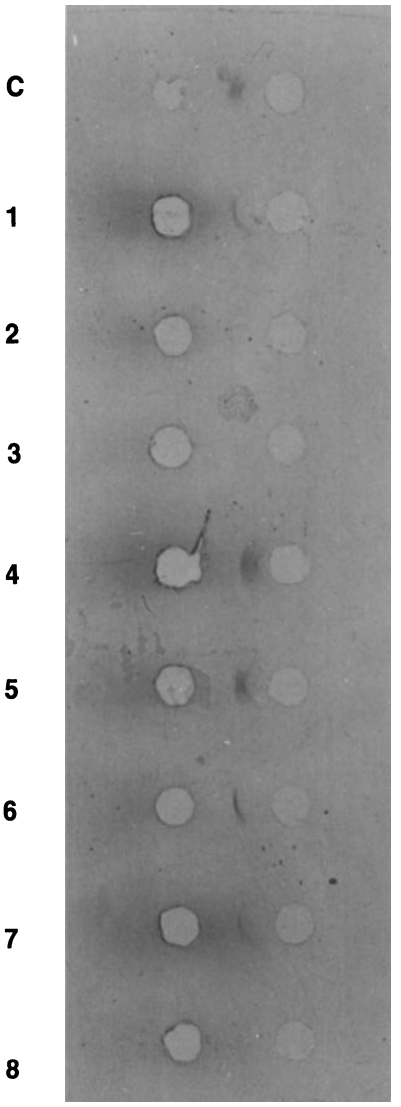

Figure 4. Titration of antitetanus toxin antibodies by counterimmunoelectrophoresis. The positive control was obtained by filling the cathodal well with tetanus toxoid at 7 Lf/ml and the anodal well with a 1/25 dilution of antitetanus toxoid (all reagents obtained from Lederle Laboratories). All cathodal wells on the plate were filled with tetanus toxoid at 7 Lf/ml. Anodal wells 1–3 were filled with undiluted, 1/2, and 1/4 diluted serum samples, respectively, of a normal, immunized child (J. D.), and wells 4–8 were filled with undiluted, 1/2, 1/4, 1/8, and 1/16 diluted serum samples, respectively, from a second immunized child (A. H.). In the first child, there was reaction only with undiluted serum; in the second child, there was an antibody titer of 1/8.

One way to increase the sensitivity of radial immunodiffusion, described by Rowe (1969) some years ago, consists of using a radiolabeled anti-γ-globulin reagent to reveal precipitin rings too weak to be seen even after staining. Radioactive radial immunodiffusion has been used for the detection of rubella antibodies (Al-Nakib *et al.*, 1975), but, again, what is gained in sensitivity is lost in technical complication.

2.2.4. Immunofluorescence Tests

The use of immunofluorescence for antibody quantitation is based on simple principles, It requires an insoluble substrate containing the antigen, a serum sample with suspected antibodies, and a fluorescein-labeled anti-Ig. Obviously, the simplest substrate is the infecting agent itself; for example, (1) the fluorescent treponemal antibody test for diagnosis of syphilis (Sussman, 1975); (2) the fluorescent tests for antifungal antibodies using the whole fungus (*Candida albicans, C. neoformans, H. capsulatum*) as substrate, in which antibodies might be detected either with a fluorescein-conjugated anti-human Ig antiserum or by inhibition of staining with fluorescein-conjugated animal antisera to the fungi (Longbottom and Pepys, 1975); (3) titration of viral antibodies, using fixed infected cells and a fluorescein-labeled antiimmunoglobulin reagent, which in special cases can be class specific. This last allows the diagnosis of congential infections by the demonstration of virus-specific IgM antibodies (Nagington, 1975). However, in this regard, caution is necessary to exclude the existence of IgM antibodies to maternal IgG that might lead to false positives. The demonstration of antibodies to several protozoal agents by the indirect fluorescence technique is an approach successfully used in trypanosomiasis, visceral leishmaniasis, malaria, and toxoplasmosis (Wilson, 1975). Similar techniques are used in the search for antibodies in helminthiasis, such as trichinosis, echinococcosis, and schistosomiasis (Soulsby, 1975).

The main incovenience of the immunofluorescence techniques using infectious agents as substrates is the difficulty in developing standardized antigen preparations for adequate quantitation of antibodies. Recently, the attention of many investigators has centered on the development of techniques more adequate for quantitation, using antigens fixed in insoluble artificial substrates and measuring the intensity of the fluorescence resulting from the exposure of the antigen–antibody coated substrates to a fluorescent antiimmunoglobulin antiserum by means of a fluorometer. The first attempts along this line were made by Toussaint and Anderson (1965), who devised a technique through which the antigen is absorbed into discs of cellulose acetate which are then exposed to serum possibly containing antibodies and to a fluorescein-labeled anti-human immunoglobulin reagent. Under these conditions, fluorescence can be quantitated by direct reading in a fluorometer. This technique was soon adapted by Gore *et al.* (1970a,b Gore and Sadun, 1968) to the diagnosis of filariasis, echinococcosis, and trichinosis, while Toussaint (1966) described an improved technique for the diagnosis of schistosomiasis. Although this technique has been used mainly in the diagnosis of parasitic infections, there is no reason why it could not be adapted to other situations, as proved by Toussaint *et al.* (1968), who used it for diagnosis of trypanosomiasis.

Other attempts have been made to find adequate substrates to couple soluble antigens for use in immunofluorescence tests. Camargo and Ferreira (1970) used cellulose particles, to which they coupled soluble antigens obtained form *T. cruzi* by cyanogen bromide treatment. More recently, Sepharose particles have been proposed as substrates for the coupling of antigens to be used in immunofluorescent techniques designed to allow the quantitation of a given antibody (Hernandez *et al.*, 1973; Van Dalen *et al.*, 1973). This technique has been found to be 10 times more sensitive than conventional radial immunodiffusion, with the advantage of not being limited to precipitating antibodies; but it is only one-tenth as sensitive as radioimmunoassay (Haaijman *et al.*, 1975). One of the main drawbacks of the technique, its time consumption, seems to have been eliminated by the construction of automated microfluorometers (Haaijman and Wijnants, 1975). Hernandez *et al.* (1973) reported its application to determination of anti-tetanus toxin, and Deelder *et al.* (1975) reported its application to the diagnosis of schistosomiasis and filariasis. Most other studies published so far deal

with purely experimental systems, with several technical improvements ranging from the use of agarose particles coated into glass slides (Streefkerk *et al.*, 1975a) to the substitution of fluorochrome for peroxidase (Streefkerk *et al.*, 1975b). Recently, a semiautomated, microcomputerized fluorometer has been introduced in the market (FIAX 100 Fluorometer, IDT, Santa Clara, California) that may prove to give a new thrust to the efforts for developing quantitative fluorescent techniques for antibody detection. In general lines, the technique is simple. Antigens are coated (usually by air drying) onto absorbent pads placed on the tip of plastic sticks. The antigen pad can be easily placed in a test tube containing the suspected serum. After incubation with serum, and rinsing, the antigen pad (to which antibodies might have bound) is incubated with fluorescein-labeled antiserum (polyvalent, or specific for IgG and IgM). Quantitation is done after rinsing, by direct insertion of the antigen disk (now having bound the first and second antibodies) into the microfluorometer. Systems for the quantitation of antiviral antibodies (To RCH panel) have been commercially introduced; and we have also developed a technique for the quantitation of anticandida albicans antibodies (Estes *et al.*, 1980) that appears to have a great potential for the diagnosis of systemic candidiasis when cytoplasmic antigens from the mycelial phase are used (G. B. Estes, M. Muñoz and G. Virella, unpublished observations).

2.2.5. Radioimmunoassay and Enzymoimmunoassay Tests

Radioimmunoassay and enzymoimmunoassay are among the most sensitive techniques that can be used to assay antigens or antibodies. They are similar in sensitivity, the basic difference being that the labeling in the case of enzymoimmunoassay is done with a purified enzyme, and the titration depends on the enzyme–substrate reaction. In the original technique described by Engvall and Perlman (1971), alkaline phosphatase was the enzyme used for labeling, and its activity was determined by following the hydrolysis of *p*-nitrophenylphosphate through the increase of absorbance at 400 nm. Other enzymes, such as peroxidase, have also been successfully used (Walls *et al.*, 1977; Ruitenberg *et al.*, 1976).

The problem inherent to both techniques is that they are expensive to develop, requiring expensive reagents and, in the case of radioimmunoassay, very expensive equipment. This renders them inadequate for general use in screening laboratories in underdeveloped countries. When available, however, they constitute the most powerful tools for the study of the immune response to infectious agents, not only for diagnostic purposes but also for the study of immunoresponsiveness.

Although radioimmunoassay has been used extensively for assays of hormones, cardiac glycosides, morphine, and other compounds, as well as for assays of IgG subclasses and IgE levels, its use in determination of antibodies has been more restricted. Basically, radioimmunoassay methods can be based on the use of labeled antigens, as in the case of a method proposed for the determination of antibodies to diphtheria toxoid (Bazaral *et al.*, 1973), or it can be used with a labeled second or third antibody. The first method is adequate only for cases where antibody response is known to be directed against restricted and well-defined antigens that are easy to purify. The second approach is more flexible, and whole microorganisms can be used as substrate. In the technique described by Brown and Lee (1973, 1974) for assay of antibodies to *E. coli, Bacteroides fragilis,* and enterococcus, whole microorganisms are used as substrate for antibody binding, and the amount of bound antibody is assayed through the use of a labeled animal anti-human Ig. This also allows the discrimination of IgG and IgM responses simply by varying the anti-Ig used in the second step. A similar principle has been applied to the assay of mumps antibodies (Daugharty *et al.*, 1973). More recently, Charlton and Blandford (1975) published a description of a method for assay of class-specific mouse anti-Sendai virus antibodies using a double-sandwich technique in which the viral antigen, coated into the wells of polyvinyl microhemagglutination plates, was exposed first to the serum of infected animals, then to rabbit antisera to different Ig classes, and finally to radiolabeled sheep anti-rabbit Ig. This was found to be a very convenient approach.

The enzyme-linked immunosorbent assay (ELISA), of more recent description (Engvall and Perlman, 1971), has been extensively ap-

plied to the measurement of antibodies in the last few years, and it appears to be a very successful method when applied to the detection of antiviral antibodies. By 1976, according to a review article published by Wisdour, enzymoimmunoassays had been developed for antibodies directed to several bacterial antigens (*E. coli* enterotoxins, O and K1 antigens; *Salmonella* O antigens; *Treponema pallidum; V. cholerae* enterotoxin and lipopolysaccharide), viruses (Hog cholera virus, rubella virus), protozoans (*Amoeba* strain HK-9, *Plasmodium, Toxoplasma gondii, Trypanosoma cruzi, Trypanosoma rhodescense,* and *Trypanosoma brucei*), and helminths (*Echinococcus granulosus, Onchocerca volvulus, Trichinella spiralis*). In the last few years, further additions have been made to the list of antibody assay tests based on enzymoimmunoassay. A very useful summary of recent developments was published as a supplement to the *Journal of Infectious Diseases* (October 1977). In brief, methods have been developed to detect antibodies to *Brucella abortus* and *Yersinia enterocolitica* (Saunders *et al.*, 1977; Carlsson *et al.*, 1976), tetanus toxoid (Stiffler-Rosenberg and Fey, 1977), Legionnaire's disease organism (Farshy *et al.*, 1978), *Rickettsia quintana* (Herrmann *et al.*, 1977), reoviruslike agents of infantile gastroenteritis (Yolkev *et al.*, 1977), influenza and parainfluenza viruses (Leinikki and Passila, 1977; Bishai and Galli, 1977); cytomegalovirus, measles virus, adenovirus, coxsackie virus (Leinikki and Passila, 1977; Bidwell *et al.*, 1977), herpesvirus (Bidwell *et al.*, 1977; Vestergaard *et al.*, 1977; Gilmam and Docherty, 1977); *Toxocara canis* (Ruitenberg and Van Knapen, 1977).

In spite of this widespread enthusiasm, some caution has to be recommended to those wishing to develop enzymoimmunoassay techniques (Bullock and Walls, 1977). Standardizations of reagents and methods is practically nonexistent; not all antigens are similarly easy to coat into polystyrene tubes; not all polystyrene tubes or microtiter plates are equally suitable for antigen coating; conjugation of enzymes into antibodies is a delicate, time-consuming, and expensive technique; avoidance of background reactions is a major problem still awaiting a clear solution. At this time, enzymoimmunoassays have been proved to be widely applicable. The near future will show whether these assays can be standardized and successfully introduced in the serological routine.

2.3. Antibody Detection in CSF

The detection of antibodies in CSF has great diagnostic importance in cases of suspected involvement of the CNS in any given infection. In principle, all techniques described for the detection of antibodies in serum can be used for the detection of antibodies in CSF. However, the antibody titers in this fluid are usually lower than in serum, and for this reason only the more sensitive techniques are usually successful. One main problem is to be sure of the origin of antibodies detected in the CSF. Although there is no doubt about the capacity of synthesis of antibodies by meningeal and perimeningeal plasma cells, there is also no doubt about the possibility of IgG antibodies crossing the meningeal barrier. In this perspective, the demonstration of IgM antibodies would, in theory, by more significant, but in the situations in which CSF antibodies have been searched for and detected, the IgM response has appeared to be undetectable or very short lived.

A good example of the application of antibody detection techniques is provided by studies published in the last 5 years concerning multiple sclerosis and subacute sclerosing panencephalitis. Interest in the antibody content in CSF arose from the verification that this fluid contained increased levels of IgG (Link, 1973), which on agarose gel electrophoresis were shown to be at least partially due to the appearance of homogeneous γ-globulin fractions (Vandvik and Skrede, 1973) (Fig. 5). With classical virological techniques, it was possible to prove, using large numbers of patients and properly matched controls, that both patients with multiple sclerosis and those with subacute sclerosing panencephalitis had significantly increased levels of measles antibodies in serum and CSF (Salmi, 1973; Salmi et al., 1973). Vandvik and Norrby (1973) were later able to isolate homogeneous IgG fractions from the CSF of some patients with subacute sclerosing panencephalitis and, by testing these fractions for their antibody content to measles virus by conventional serological techniques (neutralization, hemolysin inhibi-

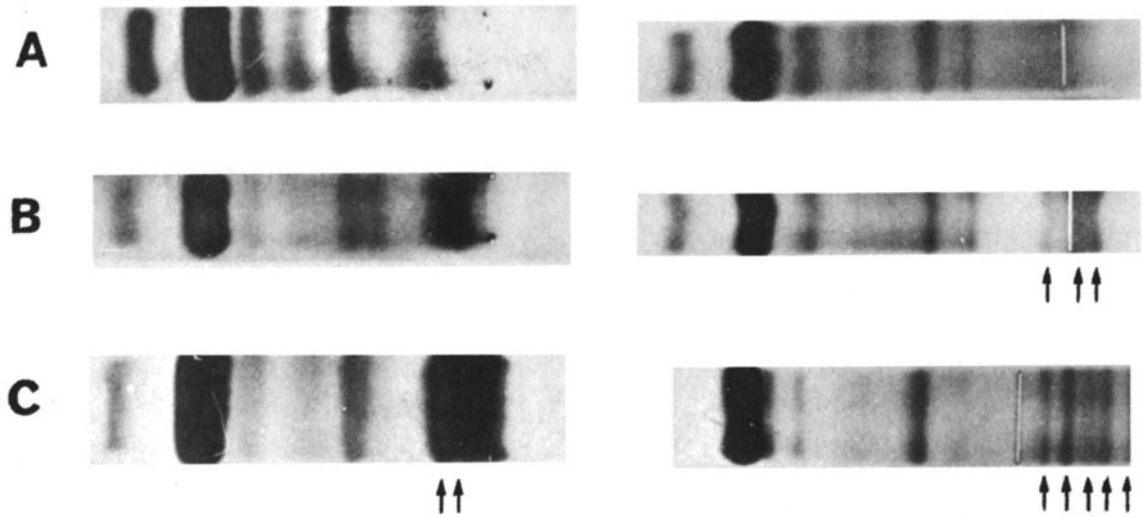

Figure 5. Types of CSF protein patterns developed by electrophoresis on cellulose acetate (left) compared with agarose gel (right). A, Normal pattern; B, a case of MS; C, a case of SSPE. In C, the agarose batch and the mold for the application slits were different from those used in cases A and B. The arrows indicate narrow gamma zones. Reproduced from Vandvik and Skrede (1973) with permission.

tion, and complement fixation), were able to conclude that these fractions represented homogeneous antibodies to measles virus. These fractions were not detectable in serum, appearing to be locally synthesized. The same group then developed a simple and easy counterimmunoelectrophoretic method which will probably replace complement fixation, to detect antibodies to the viral nucleocapsid in CSF (Nordal *et al.*, 1975). Later, through the use of radioimmunoassay with radiolabeled virus, it was possible to prove that multiple sclerosis patients also have IgM antibodies to the virus, those antibodies apparently being rather specific for the disease (Cunningham-Rundles *et al.*, 1975).

Another area in which antibody detection in CSF has proved of diagnostic relevance is in the investigation of herpes encephalitis. Antibodies to herpes simplex virus (HSV) have been investigated by classical techniques, including complement fixation, hemmaglutination, neutralization, and immunofluorescence (MacCallum *et al.*, 1974; Kurtz, 1974; Lerner *et al.*, 1972; Leventon-Kriss *et al.*, 1976). In most cases the antibodies are of the IgG type and coexist with high titers of serum IgG antibodies. IgM responses are only detectable by these techniques in rare patients (Leventon-Kriss *et al.*, 1976; Rotmensch *et al.*, 1976). However, it is accepted that the presence of HSV antibodies in the CSF and a fourfold rise in serum antibody titers, combined with a clinical picture of encephalitis, in the absence of clinical evidence of other infections of possible herpetic etiology, and a leak of blood into the CSF being ruled out, are diagnostic of herpesvirus encephalitis (Lerner *et al.*, 1972; MacCallum *et al.*, 1974). The importance of reaching the diagnosis in this way is stressed by the fact that the other alternative is the detection of HSV in brain tissues obtained by biopsy (Flewett, 1973; Leider *et al.*, 1965).

It seems possible that similar approaches might prove diagnostically relevant in other forms of meningoencephalitis of obscure etiology. However, at the present time, this is still an area of investigation and future development.

3. Antibodies in Mucosal Secretions

It has been clearly demonstrated that the antibody-mediated protection of human mucosal surfaces resides predominantly in the secretory immunoglobulin A system (S-IgA) (Tomasi, 1972; Menzel and Rowley, 1975; Crawford *et al.*, 1975; Nakajima *et al.*, 1975). Different plasma cell clones synthesize approximately equal amounts of IgA and IgG,

but a substantial percentage of IgA, in the form of dimeric secretory IgA, is lost from the systemic fluids and secreted as secretory IgA (S-IgA) through the mucosa or via exocrine glands onto mucosal surfaces (Coelho *et al.*, 1974; Rádl *et al.*, 1975).

The antibody-producing machinery responsible for the production of S-IgA is localized to the lymphoid tissues at submucosal sites, i.e., the gut- and bronchus-associated lymphoid tissues (for review, see Bienenstock *et al.*, 1975).

In our laboratory and in other laboratories (Strober *et al.*, 1976; Brandtzaeg, 1975a), investigators speculate that two parts of the S-IgA molecule, the secretory component (SC) and the J chain, hold a portion of the "localization key" to the IgA system (Sletten *et al.*, 1975; Koshland, 1975; Mestecky *et al.*, 1974; Hauptman and Tomasi, 1975). Secretory component is the only part of an Ig molecule that is known not to be produced by plasmocytes. This 80,000 molecular weight component is produced by epithelial cells in the mucosa and exocrine glands. The strong affinity of SC for the Fc region of polymeric immunoglobulins such as dimeric IgA is thought to be due to the presence of J chain, which serves to "join" the monomeric Ig units.

Since bone-marrow-derived (B) lymphocytes and plasma cells committed to S-IgA production make the dimeric IgA that contains the J chain, an interaction with the mucosal epithelial cell product (SC) might be responsible in part for the concentration of S-IgA-producing cells at submucosal surfaces, the exocrine glands, and lymph nodes draining mucosal areas. Secretory component may also be responsible for the transport of dimeric IgA through the epithelial cells and for the concomitant protection of the S-IgA molecule against proteolytic digestion. Individuals who are deficient in IgA often secrete IgM onto the mucosal surface, and IgM is also found to occur naturally in colostrum. The IgM replacement may be secondary to noncovalent binding of SC to polymeric IgM molecules which contain the J chain (Weiker and Underdown, 1975; Eskeland and Christensen, 1975; Brandtzaeg, 1975b) and the sequence of events that occur as seen with J-chain-containing dimeric IgA. The molecular mechanisms responsible for induction or regulation of the biosynthesis of SC and the overall effect of the levels of SC on the S-IgA secretion rate are unknown.

The possible regulatory role of thymus-influenced (T) lymphocytes in the production of IgA and the influence of IgA-coated antigens is interesting in terms of control and function of mucosal IgA responses. Recent findings (Koshland, 1975; André *et al.*, 1975) lead us to suggest that lymphocytes may have receptors which recognize J-chain-containing immunoglobulin. The molecular nature of the receptor(s) is unknown, but it would be exciting to postulate that a molecule with a receptor for J chain or secretory component could be present on T lymphocytes or a subpopulation thereof.

Further suggestive evidence for the critical involvement of T lymphocytes in IgA production lies in studies by André *et al.* (1975), O'Neill and Romsdahl (1974), and Atwater and Tomasi (1978). Tolerance to IgA-coated antigens is likely facilitated through an interaction between S-IgA immune complexes and T lymphocytes, thus explaining the lack of secondary IgA responses, or pointing out an important suppressor (feedback inhibition) function of T lymphocytes in the secondary IgA response. It is known that neonatal thymectomy in a variety of animals induces IgA deficiency, again implicating the thymus and T lymphocytes in regulating IgA production (Lamm, 1976). Recent studies have shown the importance of T lymphocytes in the cell-mediated immune defense systems for mucosal surfaces. The IgA system may be the most important antibody system for protection of mucosal surfaces in other ways not previously considered; for example, the interaction of T lymphocytes with IgA-antigen complexes could theoretically produce both their cellular chemotaxis near infected submucosal areas and their simultaneously enhanced stimulation in that area.

These considerations of T lymphocyte involvement in IgA production are speculations but are not entirely unjustified considering the evidence tying these two systems together. It is possible that the regulatory lymphocytes which are associated with the IgA system may represent a subpopulation of T lymphocytes with receptors for molecules containing J-chain or secretory component. Therefore, a second point that should be mentioned in re-

gard to the use of mucosal antibody titers or total S-IgA levels for diagnostic purposes is that secondary IgA responses to mucosal antigenic stimulation are low, and in many cases the increase in titer is undetectable. The reason for the poor secondary response to antigens in the presence of specific IgA is believed to be due to both T-lymphocyte induction of tolerance or suppression to the antigen and the mucosal adherence inhibition properties of S-IgA (i.e., once antigens are coated with specific S-IgA, their adherence to mucosal epithelial cells may be mitigated). Of course, secondary responses to the antigens cannot be generated without a tissue-penetrating secondary stimulus; hence elevated secondary IgA responses to the same mucosal antigen might not be detected (André *et al.,* 1974).

Since dimeric-IgA-producing cells with J chain have a "tendency" to be stimulated by mucosal antigens and then home back to "all" of the mucosal tissues, with longer times required for more distant sites, it is not surprising that, in guinea pigs, tear fluid antichlamydial S-IgA is found after vaginal infection, or that, in dogs and rabbits, oral immunization with live *Vibrio cholera* induces high titers of IgA antibodies in the mammary secretions at a later time (Brandtzaeg, 1974). In many of the above situations, but, it should be emphasized, not in all cases (Ogra and Karzon, 1971) where infection or immunization occurs on one mucosal surface, S-IgA antibody levels are also seen on distant mucosal sites (Montgomery *et al.,* 1974).

During the early neonatal period, mucosal immunocompetence is poorly developed. In fact, a "pseudo" S-IgA deficiency can be induced in germ-free animals simply due to the lack of stimulation of the mucosal immune system. Obviously, this same paucity of mucosal antigens exists in the fetus; the mucosal immune system will not have been stimulated to a stage at which the newborn infant will be completely protected from environmental pathogens. Thus the passive transfer of immune components (humoral and cellular) from human colostrum and early milk to the external mucosal surfaces of the infant's gastrointestinal and upper respiratory tracts becomes an important consideration. The decision to breast feed may be critical to the health and well being of the infant, particularly in the underdeveloped countries where a combination of malnutrition and unsanitary living conditions exist. In these countries, infant formula is often diluted in contaminated water, at times so diluted that even the nutritional value is questionable. However, human colostrum and human milk can provide a sterile, nutritious, and immunologically complete diet for the infant and can satisfy the time and nutritional requirements needed for a normal flora to develop and stimulate the development of lymphoid tissue in the submucosal areas along the respiratory and gastrointestinal tracts.

Moreover, since the mother is exposed and immunized against the local pathogens, her selective mucosal immune resistance will be temporarily transferred via the human colostrum and milk to the infant in the form of specific secretory IgA and, perhaps, specific lymphocytes.

From animal studies, it has been demonstrated that IgA-producing plasma cells in the mammary gland likely originate from precusors in mesenteric lymph nodes and gut-associated lymphoid tissues. Indeed, several observations suggest that various antigenic specificities of milk secretory IgA reflect prior antigenic encounters in the intestinal tract. For example, in human milk, neutralizing antibodies against the enterotoxins of *Escherichia coli* and *Vibrio cholerae* have been detected. In terms of meaningful immunological protection, it is significant that the highest concentrations of immunoglobulins, particularly the IgM and IgA classes, is in the early colostrum during the first 3–4 days postpartum. The IgM levels range from 27 to 30 mg/g protein and the secretory IgA levels from 22 to 35 mg/gm protein (Ogra and Ogra, 1978a). Also, the highest concentrations of lymphocytes (B and T) and macrophages (10^6 cells/ml) is observed on the first day postpartum, decreasing tenfold by the fourth day (Ogra and Ogra, 1978b).

In general, mucosal glandular secretions such as parotid saliva and submandibular saliva contain very low levels of complement components. However, in colostrum, later complement component activities many range up to 7% of those in sera (Nakajima *et al.,* 1977). The anticomplementary substances and low ionic strength effects present in mucosal secretions (Boackle *et al.,* 1978a) lead to a situation where the classical complement path-

way is blocked, yet an alternative pathway involving C3 is allowed to function (Boackle *et al.*, 1978b). The biological significance of this regulation may be in the fact that secretory IgA, when aggregated, has the ability to react with the later complement components (C3–C9), probably through a modified alternative complement pathway (Boackle *et al.*, 1974). In mucosal secretions, there are substances which directly bind to C3 and C4 (Williams *et al.*, 1975, Price *et al.*, 1975, Boackle *et al.*, 1978b). Perhaps these phenomena represent a modification or regulation of the pathways of complement activation which are present only in the mucosal secretions. Furthermore, penetration of antigens coated with complement-reactive factors might very well be partially involved in eliciting a particular type of immune or immunopathological response on infected mucosa, especially those reactions which involve the cooperation of the complement system.

3.1. Effects of Systemic or Mucosal Infection: Dependence on the Route of Infection

As eloquently pointed out by Heremans (1974) and by Ogra and Morag (1975), the route of immunization or infection appears to determine whether the immunoglobulin response is predominantly systemic or mucosal. With injected antigens or with infecting agents that replicate and disseminate in the inner body's tissues and intravascular fluids, a systemic response is seen. In these cases, the serum IgA response is variable in onset, level, and duration compared with the higher and more consistent response detected in IgM and IgG levels (Spiegelberg, 1974; McFarlane, 1973). Examples of this low, variable serum IgA response when antigens have been injected or administered systemically have been observed by Bandilla and McDuffie (1968) with *Limulus* hemocyanin; by Turner and Rowe (1964) with a paratyphoid vaccine; by Bellanti *et al.* (1967) using killed or live *Francisella tularenis;* by Smith *et al.* (1967) using virulent or killed parainfluenza virus; by Bellanti *et al.* (1967) using virulent or killed parainfluenza virus; by Bellanti *et al.* (1969) and Scott *et al.* (1972) with virulent or attenuated adenovirus; by Ogra *et al.* (1971) with a virulent echovirus; by Tokumaru (1966) with a herpes simplex virus; and by Ogra and Karzon (1971) using an attenuated or killed polio vaccine (Table 4). When contact with antigen is not systemic but is made solely by way of a mucosal surface (e.g., the digestive tract), the antibody appearing in the serum is largely composed of IgA, with IgG and IgM showing a relatively smaller response.

3.2. Problems Associated with Diagnosis of a Mucosal Infection by Measurement of Specific Serum IgA Antibody

1. High-affinity IgG antibodies (Mouton *et al.*, 1970) may mask serum IgA responses.
2. The biological half-life of IgA is shorter than that of IgG (Heremans, 1974; Smith *et al.*, 1976).
3. Depending on the physical properties of the antigen or its infectivity, high concentrations of antigen may actually penetrate normal mucosal barriers, such as the tips of the intestinal villi, and reach the bloodstream, causing both systemic and mucosal immunization.
4. Primary serum IgA responses after a mucosal stimulation are usually short lived (André *et al.*, 1975; Waldman and Ganguly, 1975).
5. The degree of induction of "tolerance" or "suppression" of lymphocytes in response to IgA–antigen complexes (André *et al.*, 1975) has not been qualitatively or quantitatively examined. The final effect on the IgA secondary response is unknown.
6. The "normal" rates of synthesis of total and/or specific IgA, IgG, and IgM at various submucosal sites has not been thoroughly investigated (Menzel and Rowley, 1975; Beeken and Roessner, 1975).

In summary, diagnostic measurement of serum IgA antibody levels can be useful for diagnosis of mucosal infection, but the above problems must be considered.

3.3. Mucosal Secretions as Future Diagnostic Fluids: Some Thoughts on Specificity

In cases of protracted mucosal infections, physicians may wish to know whether the patient is responding with production of a mu-

TABLE 4. Serum and Secretory Immunoglobulin Responses to Viral Antigens

Infectious viral agent	Route of immunization or infection	Form of antigen	S-IgA response (respiratory tract)	Serum antibody	Reference[a]
Influenza	Intranasal	Active or inactive	High	Not reported	1–4
	Parenteral	Inactive	Low		
Parainfluenza	Intranasal	Active or inactive	High	Not reported	5–8
Measles	Parenteral	Inactive	Low	Not reported	5,9
	Subcutaneous	Inactive	High		
	Subcutaneous	Active	Low		
Rubella	Natural infection or intranasal (RA 27/3)	Active	High	Not reported	10–13
	Parenteral (HPV-77)	Inactive	Low		
Polio	Oral	Attenuated (Sabin)	High	High	14,15
	Intranasal	Inactivated (Salk)	High	Not detectable	
	Parenteral	Inactivated (Salk)	Low	High	
Herpes type II	Vaginal	Infectious	High	Low	16

[a] References: 1, Waldman (1969); 2, Waldman *et al.* (1969); 3, Dayton *et al.* (1971); 4, Shvartsman and Zykov (1976); 5, Bellanti *et al.* (1969b); 6, Smith *et al.* (1967); 7, Smith *et al.* (1966); 8, Smith *et al.* (1976); 9, Joseph *et al.* (1975); 10, Ogra (1973); 11, Ogra *et al.* (1971); 12, Plotkin *et al.* (1973); 13, Al-Nakib *et al.* (1975); 14, Ogra and Karzon (1971); 15, Ogra *et al.* (1968); 16, Dent and Bienenstock (1974).

cosal antibody. In regard to microbial agents which could produce repeated localized mucosal infections such as gonorrhea, trachoma, and mycoplasma, it might be of diagnostic advantage to measure the level of specific secretory IgA in the local mucosal secretion. Work by O'Reilly *et al.* (1976) indicates that, whereas both serum and cervicovaginal secretions can be used to indicate that a gonorrhea infection has occurred, the short-lived nature of the mucosal S-IgA immune response may be of better diagnostic advantage in indicating the actual presence of an active infection. It is important to realize with regard to gonorrhea that other neisseria species are normal mucosal inhabitants (i.e., in the oral flora) and they may induce low levels of cross-reacting serum and mucosal IgA antibodies even in normal individuals. In using mucosal secretions for standard qualitative and quantitative determinations of antibody levels (Cowan, 1973), certain concepts regarding specificity should be remembered. It has been stated by some investigators that after influenza virus infection the secretory IgA response, in contrast to the serum antibody response, presents a broad spectrum of antigenic specificities, cross-reactive to different extents with the initial antigenic stimulus. This diverse specificity may be of biological advantage to the host since it would afford a degree of cross protection against other related pathogens and microbial products. Theoretically, this broad response may be effected by bystander B cells in submucosal sites which are "nonspecifically" activated to differentiate to appropriate immunoglobulin-producing plasma cells by specific T lymphocytes undergoing mitogenic stimulation near the mucosal infection. Henney (1975) has recently reviewed the immunological importance of lymphokines produced directly by activated T and B lymphocytes in the protection of mucosal surfaces of the lung.

Diagnostic utility might be derived from the cross-reactivity occurring in mucosal secretions during or after infections. For example, Larson *et al.* (1976) have speculated that antibodies to rabbit erythrocytes are consistently present in saliva (and serum) because the rabbit erythrocytes carry at least one antigenic determinant cross-reacting with some antigen to which humans are continuously exposed. The same authors further postulated that hemagglutination of rabbit erythrocytes by saliva may prove a useful and simple test for quantitating levels of total secretory IgA. It should be kept in mind that results of this test may be misleading in individuals who are selectively deficient in IgA; these individuals may have substitutive levels of IgM and IgG agglutinins.

By far the greatest handicaps in diagnosing mucosal infections using mucosal antibody levels are the difficulties in standardization and errors of measurement (Heremans and Masson, 1973). For example, salivary S-IgA levels vary as a function of flow rate. Before a standardized value is obtained, several salivary collections may be necessary. Also, the calibration standards influence the final calculated level to varying extents depending on the quantitative method used. A nephelometric method for the preparation of saliva samples for immunoglobulin analysis has been published by Virella *et al.* (1978). Complement-fixation tests are presently not applicable because of the unknown effects of complement-reactive proteins in saliva and other mucosal secretions (Boackle *et al.*, 1978a).

Radioimmunoassay (Beeken and Roessner, 1975), radial immunodiffusion, and virus-neutralizing antibody tests for jejunal and nasal secretions are commonly used in research laboratories (Bellanti and Artenstein, 1964), and routine clinical laboratory tests utilizing these secretions may appear in the near future. Specificity of secretory antibodies to bacterial immunogens can be quantitated by enzyme-linked immunoassay, by passive hemagglutination, and by bacterial agglutination techniques (Ebersole and Molinari, 1976). When using passive hemagglutination, care must be taken to control for agglutinins to sheep and rabbit erythrocytes in mucosal secretions (Chauncey *et al.*, 1966), as well as for enzyme-exposed antigens (e.g., neuraminidase-exposed T antigens).

3.4. Diagnostic Tests for Determination of IgA Deficiency

The many papers on IgA deficiency suggest a possible increase in various clinical symptoms, including predisposition to respiratory, gastrointestinal, and other mucosal infections (Michel *et al.*, 1975). The production of anti-α-chain antibodies and other autoantibodies [e.g., anticollagen antibodies resulting in collagen diseases (Koistinen, 1975; Koistinen and Sarna, 1975; Ogra *et al.*, 1974)] has been noted in IgA-deficient patients.

The influence of cell-mediated immunity (T lymphocytes) in mucosal protection, both directly via lymphokine production and indirectly via cooperation with B lymphocytes to stimulate their differentiation into IgA-producing plasma cells, is an important entity which should be tested in IgA-deficient individuals who suffer recurrent infections. Some IgA deficiencies develop because of a defect in T lymphocytes (or T-lymphocyte cooperation), and a significant proportion of IgA-deficient patients do have B cells which have α-chain antigens on their surface but lack the ability to differentiate into IgA-producing plasma cells *in vivo* (Lawton *et al.*, 1974). Saliva, particularly from the parotid glands, may be a useful diagnostic fluid for the determination of levels of S-IgA on mucosal surfaces (Virella *et al.*, 1978; Emmings *et al.*, 1975). In normal individuals, the average levels of S-IgA in parotid saliva generally reflect the levels of S-IgA present on the other mucosal surfaces.

The use of concentrated pure parotid saliva avoids the bacterial enzymes (Plaut *et al.*, 1974, 1975) present on mucosal surfaces and in whole saliva, which have recently been found to cleave the Fc region from the secretory IgA molecule (the IgA1 subclass). Whether bacteria take advantage of this IgA destruction is not known. Methods for the collection of parotid saliva have been described by Shannon and Chauncey (1967) and by Rossen *et al.* (1971).

The low-level radial immunodiffusion test (Mancini *et al.*, 1965) or nephelometric analysis (Virella *et al.*, 1978) utilizing rabbit antiserum specific for the alpha chain of S-IgA should be the first test employed for determining levels of serum or secretory IgA (Heremans and Masson, 1973). If this procedure fails to demonstrate alpha-chain antigens, the more sensitive passive hemagglutination inhibition test or radioimmunoassay should be used (Koistinen, 1975). Goat antisera should be avoided in the above determinations because IgA-deficient subjects might produce IgM or IgG antibodies cross-reactive with goat proteins.

Lack of IgA in the saliva, together with elevated or normal levels of serum IgA, may represent indicators of a newly found disorder, secretory component deficiency (Strober *et al.*, 1976; Ogra *et al.*, 1975). An elevated number of B cells producing IgA are found in the peripheral blood, indicating that these cells may lack the ability to "home" into the mu-

cosal sites where the SC "receptors" are present.

In summary, the most useful clinical information one can obtain in diagnosing recurrent mucosal infections is to test for presence or absence of IgA and specific IgA antibodies in mucosal secretions and to couple that information with determination of the levels of total IgA and specific IgA antibody in the serum. In-depth information on the immunological mucosal response of a patient to an infection must relate specific serum and mucosal IgA antibody levels with T-cell responsiveness to the antigen in question.

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