Specific Analysis of Immunoglobulins.
Techniques and Clinical Value

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We recommend that routine screening of sera for immunoglobulin disorders consist of a combination of electrophoresis in agarose and immunochemical quantitation of serum IgG, IgA, and IgM. Abnormalities detected by this procedure may be classified as (a) immune deficiencies, (b) polyclonal hyperimmunoglobulinemias, or (c) monoclonal immunoglobulin disorders. The analyst can contribute to the diagnosis of immune deficiency by detecting evidence for plasma protein losses in the urine, unsuspected monoclonal immunoglobulin disorders, and abnormalities in the immunoglobulin content and spectrum of secretions. Polyclonal hyperimmunoglobulinemia—seen in infections, autoimmune disease, liver disease, sarcoidosis, or other conditions—is frequently associated with an imbalance between the different immunoglobulin classes, and with the appearance of discrete inhomogeneities in the immunoglobulin zones of the electrophoretic spectrum. Factors involved in this process are listed, and examples are indicated. Monoclonal immunoglobulin disorders may not be detected if the tumor protein is masked by normal serum-protein fractions or misdiagnosed by confusion with nonimmunoglobulin components. Diagnosis of immunocyte malignancy is aided by detection of Bence Jones protein in the urine; measurements of serum IgG, IgA, and IgM concentrations; and monitoring the concentration of the abnormal component in the serum as the disease evolves.

Additional Keyphrases: IgG, IgG, IgM • Bence Jones protein • diagnostic aid • Electrophoresis in agarose gel • routine screening for immunologic disorders • electrophoresis of urine

Methods to explore and assess quantitatively the different components of the immunoglobulin spectrum are available in a bewildering assortment that may confuse both analyst and clinician. Evidently, an elementary battery of a few simple tests is all that is needed for adequate screening; when necessary, these tests can of course be complemented by any amount of sophisticated investigation that the situation may require.

Here, we propose such an all-purpose immunoglobulin screening program, one adopted in our laboratory after 20 years' experience with nearly every method proposed in the field.

Techniques

Nearly every type of immunoglobulin disorder can be detected and assessed by a judicious combination of agarose-gel electrophoresis of the serum proteins, and quantitative immunochemical determination of the three major immunoglobulin classes: IgG, IgA, and IgM.

IgD is excluded from this analysis because it has no clinical significance at present, and because of the difficulty inherent in its determination. IgE, which can be measured by radioimmunoassay (1, 2), is omitted from the routine panel, because increased concentrations are predictable in reaginic allergy (3, 4) or intestinal parasitic disease (5) and its determination does not contribute to other diagnoses, except IgE myeloma.

Electrophoresis

The technique we adopted is that of Laurell and Johansson (6), by which 6 μl of serum is run for 45 min in a 1-mm thick layer of agarose (Industrie Biologique Française, Gennevilliers, France)—10 g/liter of barbital-sodium barbital buffer, 0.075 mol/liter, pH 8.6, containing 2 mmol of calcium lactate per liter—in a water-cooled apparatus of our own construction. Staining is with Amido Black. Apparently, cellulose acetate is about equally useful; in this case the macrosystem recommended by Kohn (7) has proved more satisfactory than the micro-method used in many laboratories. A drawback with cellulose acetate, as with any noncooled supporting medium exposed to free evaporation of the buffer, is the difficulty in controlling the length of the pattern and its position relative to the application point. This may cause serious inconvenience when it is desired to compare samples collected from the same patient run at different times, as is frequently necessary in monitoring the evolution of an immunoglobulin disorder.

The electrophoretic pattern for plasma is more informative than that for serum, because the former includes fibrinogen as well as those components of complement that are easily damaged by the proteolytic enzymes activated during blood clotting (8). Nevertheless, serum is to be preferred when the pur-
pose is to screen the immunoglobulin spectrum, because the fibrinogen band is likely to obscure, or cause confusion with any electrophoretic immunoglobulin abnormality lying in a beta-2 region. In fact, the relative importance of the IgA fraction is evaluated from the density of the beta-2 area (in Figure 1, compare samples No. 2703 and 2829—with high IgA—and samples 2733 and 3044—with low IgA—to the normal control, 2713). No such deductions can be made from the electrophoretic pattern as far as the IgM content is concerned, so that separate IgG, IgA, and IgM determinations are in any case mandatory.

**Format of the report.** In many laboratories it has become ritual to provide the clinician with a quantitative evaluation of the different electrophoretic protein fractions, or at least with a graph of the scan. There is no justification in doing so, particularly not in the case of the immunoglobulins, whose different classes have overlapping mobilities and cannot be distinguished by such criteria. A second reason militating against scanning is that the analytical power of the eye is far superior to that of any scanning device in detecting discrete monoclonal components standing out on a heavily stained background. To bring these out would require the use of image-processing techniques such as are commonly used in space research or work with the electron microscope, but which have not to this date been adapted to graphic analysis.

We therefore prefer to provide the clinician with a half-tone picture of the stained run, a duplicate of which may also be filed when the storing of vast quantities of glass plates is undesirable. At this laboratory it is standard practice to run nine samples side by side on each 11.0 × 20 cm glass plate. “Polaroid” instant pictures (8.5 × 10.5 cm) are made of the stained plates and these are cut into individual patterns, which are issued with the protocol. Typical photographs from such plates are shown in Figures 1 to 5.

However, there is one case in which a quantitative scan of the run is important: monoclonal components, the concentration of which must be calculated from the combined data of total protein determination and planimetric evaluation of the scan (see “Evaluation”).

Electrophoresis of concentrated urines is also an important step in the diagnosis of monoclonal immunoglobulin disorders. Here, a picture of the run with a parallel sample of serum from the same patient is all that is needed, except a value for urinary total protein. With enough albumin or other proteins present in the sample, scanning can be contemplated if accurate values are desired.

**Immunoochemical Quantitation of IgG, IgA, and IgM**

Until recently, these determinations have been carried out by single radial immunodiffusion, accord-

![Fig. 1. Electrophoreses in agarose gel (pH 8.6) of sera with various concentrations of the three main immunoglobulins, A, M, and G](image-url)
The results may be expressed in absolute terms (g/liter) or as percentages of the mean normal value for the population concerned. We prefer the latter system, because the problem of standards is completely removed, so that data become comparable from one laboratory (in the same geographic area) to the next, and also because the clinician is spared the effort of interpreting the results with respect to a normal range with which he may not be familiar. For instance, Michaux (16) found the "normal" IgG serum concentration among the African staff of a hospital in Kinshasa to be nearly twice that of the Europeans employed in the same institution, and noted that, in the former, major differences existed depending on sex and general standard of hygiene.

Evaluation

The immunoglobulin abnormalities detectable by routine tests may be ordered under three headings: (a) immunoglobulin deficiencies, (b) polyclonal hyperimmunoglobulinemias, and (c) monoclonal immunoglobulin disorders.

Immunoglobulin Deficiencies

Combined immune deficiencies—bearing on more than one immunoglobulin class and frequently on cell-mediated immunity as well—may arise through abnormal renal or intestinal losses, by the effect of excessive catabolism, or by any of the poorly elucidated mechanisms that impair the biosynthesis of antibodies. The ultimate diagnosis will frequently require a complete immunologic investigation of the patient, or at least metabolic studies on his immunoglobulins (17). There are, however, some precise contributions that the clinical analyst can make or suggest.

Firstly, immunoglobulin deficiency owing to renal losses can be diagnosed from its association with very low serum albumin and elevated alpha-2-macroglobulin (visible as a pronounced fast alpha fraction), as well as elevated very-low-density lipoprotein (appearing as a wavy sharp band behind the beta-1 band of transferrin), which are the other landmarks of the nephrotic syndrome (Figure 2).

Secondly, some cases of combined immune deficiency in the adult are in fact expressions of a lymphoma. It is therefore recommended that 100-fold concentrated urine be screened for Bence Jones protein, and that the electrophoretic serum protein spectrum be scrutinized for faint monoclonal immunoglobulin bands. Since these may hide among the major protein fractions, even as far anodally as albumin in the case of certain serum Bence Jones proteins, this would be one of the really mandatory indications for two-dimensional immunoelectrophoresis (18).

Selective immunoglobulin deficiencies are one of the major justifications for including routine IgG, IgA, and IgM determinations in the analysis, because, as discussed before, the electrophoretic pattern tends to tell us nothing about them. The following specific recommendations should be kept in mind.

Firstly, a quantitative assay of different antibody activities—isoagglutinins, antistreptococcal—should be carried out or recommended, because some immune deficiencies are characterized by an unbalanced spectrum of antibody activity of the serum immunoglobulins rather than by gross deficiencies in their overall level (19).

Secondly, clinically useful information can be secured by determining the presence or absence in certain secretions—not the absolute concentrations, which are too difficult to measure—immunoglobulins IgA, IgM, and, perhaps, IgE. Tears are easily obtained from children, and nasal fluid is collected by instilling 5 ml of saline into the nostril with the head leaning backwards and glottis closed, and then having the patient expel it into a basin. Saliva is readily obtained, but must be centrifuged free of food and bacteria before shipping. All fluids should be preserved with thimerosal or, as a useful substitute, chloramphenicol. It is particularly interesting that many patients with selective IgA deficiency have in their secretions considerable amounts of IgM, whereas almost none is found in the normal (20).

Finally, any immune deficiency not caused by losses of plasma proteins should warrant a search for similar cases among the patient's relatives.

Polyclonal Hyperimmunoglobulinemia (Secondary Hypergammaglobulinemia)

The apparent electrophoretic continuity of the beta-2 and gamma areas of the normal pattern, where the bulk of the immunoglobulins is confined, is merely an illusion, comparable to that of the
seemingly continuous color spectrum produced by a poorly resolving spectrocope. Electrophoretic techniques simply cannot resolve the hundreds of thousands of tiny individual protein bands that occupy these areas, each of which represents a discrete chemical species of antibody. This multiplicity is created by the fact that each "antigen" in the serological sense (for instance a molecule of foreign protein) is itself a mosaic of different antigenic determinants, and that each determinant is capable of inducing many different types of antibody molecules, each having its own electrophoretic mobility. Each of these antibodies is assumed to be synthesized by a separate clone of immunocytes, after stimulation by the antigen.

Polyclonal hyperimmunoglobulinemia (Figure 3), also known as secondary hypergammaglobulinemia, is the condition that commonly arises after extra stimulation of the immune defenses. The offending antigen(s) may be known (as in infectious diseases), suspected or under dispute (as in autoimmune disorders), or difficult to define at all (as in subacute and chronic liver disease or sarcoidosis), which are the four main diagnoses to be considered in such cases.

The polyclonal origin of secondary hypergammaglobulinemia does not necessarily mean that all immunoglobin classes and all corresponding electrophoretic fractions will undergo a parallel increase. Very frequently this increase is quite unbalanced (Figure 1) and in the extreme case discrete antibody bands may make their appearance amidst the diffuse background, so as to pose a problem of differential diagnosis with monoclonal immunoglobulin disorders (Figure 4) (see below). A list of factors favoring such restricted heterogeneity of the immune response is presented in Table 1. In some cases, the nature of

Table 1. Factors Responsible for Restricted Heterogeneity of the Immune Response

<table>
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<tr>
<th>Factors</th>
<th>Examples</th>
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<tbody>
<tr>
<td>1. Factors involving the antigen.</td>
<td>Antibodies to blood-groups A1 (32) or I (33, 34). Restricted heterogeneity Ig patterns in acute viral diseases?</td>
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<tr>
<td>Nature of the antigen (few antigenic determinants or special steric requirements for fitting of antibody)</td>
<td>Restricted heterogeneity (pseudomonoclonal) patterns in immune deficiency, especially after marrow graft (21) Restricted heterogeneity and IgM predominance in infections of fetus or newborn.</td>
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<td>2. Factors involving the host (immunological immaturity: few clones available)</td>
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<td>3. Factors involving host-antigen interaction</td>
<td>Mucosal portal of entry (favoring IgA response) Rise of serum IgA during urinary infections.</td>
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<td>(a)</td>
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<td>(b)</td>
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Fig. 3. Electrophoreses in agarose gel (pH 8.6) of sera from patients with polyclonal hyperimmunoglobulinemia, compared to serum from a normal person (NI) In: serum from a patient with pulmonary infection. Note the increase of haptoglobin (Hp) CI: serum from a patient with cirrhosis. Note the increase of α1-antitrypsin (AT) and the decrease of haptoglobin (Hp); the bands of α2-macroglobulin (α2M) and haptoglobin should not be confused.

Fig. 4. Electrophoreses in agarose gel (pH 8.6) of sera showing oligo- or monoclonal bands (arrows) NI, normal serum; A: gastro-intestinal infection in a newborn; B: viral hepatitis; C: cirrhosis The band of C-reactive protein (CRP) should not be confused with those of monoclonal components After storage at 4°C for 2–3 days, the samples show a band in the β2 region that may mimic a monoclonal component. It corresponds to a degradation product of β1C (C3 factor of complement) appearing before the formation of β1A.
the antigen may be the sole factor involved, but the responsibility usually lies with the host or with the way the antigen has been dealt with in the body. The most striking case is certainly that where an apparently monoclonal band puts in a lone appearance in a serum devoid of immunoglobulins, during reconstitutive cell therapy of combined immune deficiency (21); later this band is seen to fade and be replaced by a more conventional diffuse gamma zone (21). This phenomenon is obviously due to the fact that at the onset of reconstitution only one or at most a few immunocyte clones were able to respond to the antigenic stimulation from the surrounding medium. In lesser proportions an analogous mechanism may be thought to account for the restricted heterogeneity pattern (Figure 4) and predominant IgM concentration (22) commonly found in fetuses or newborns that are sustaining a viral, toxoplasmal, or bacterial infection. Also very striking, and probably similarly explained by competition being staged amongst only few clones, is the well-documented banding so conspicuously present in the cerebrospinal fluid immunoglobulins of patients with autoimmune or chronic infectious disorders of the brain (Figure 5) (23). This is one of the two cases in which the anatomic localization of the antigen in the body has a pronounced effect on the antibody distribution in the blood; the other case in point is the observation that mucosal infections—for instance, of the urinary tract—may give rise to a selective increase of the IgA level in the blood (Figure 6), presumably because the mucosa are a site of predominant IgA antibody synthesis that, moreover, contributes IgA to the circulation (24).

Restricted heterogeneity of the immunoglobulin area cannot be discerned with any of the less resolving methods of electrophoresis, which is a major reason for recommending agarose gel electrophoresis, as outlined in the technical section. Two sources of error to be borne in mind while diagnosing this condition are, firstly, genuine monoclonal bands typical of immunocytoma (see below) and, secondly, the faint mid-gamma band corresponding to C-reactive protein (Figure 4), which is likely to be visible in sera from patients with acute inflammatory conditions (25). However, the context, in this case, is always typical of the inflammatory syndrome, i.e., through the concomitant rise of the alpha-globulin fractions (Figure 4). In acute hepatitis sera a faint gamma band will often be visible (Figure 4), only to vanish when the condition improves. The true status of this component is not yet clear.

Monoclonal Immunoglobulin Disorders (Immunocytoma)

Tumors of immunoglobulin-producing cells (immunocytomas) are generally classifiable either as multiple myeloma (Kahler's disease) or Waldenström's macroglobulinemia, but other varieties exist as well (26, 27). In myeloma the tumor product is usually IgG or IgA, seldom IgM, and very rarely IgD or IgE. Bence Jones proteins, consisting of incomplete molecules comprising only light polypeptide chains, are commonly found in myeloma and more rarely in macroglobulinemia, and are better observed in the urines, where they become enriched.

In some cases no immunoglobulin is synthesized, or secreted, by the tumor cells, and these cases may mimic immune deficiencies arising from other causes. Usually, however, the secretion product from the tumor cells shows up in the serum or urine as a narrow band superimposed on the diffuse background of normal antibody immunoglobulins, and hardly poses any problems of diagnosis (Figure 7). As already mentioned, a monoclonal immunoglobulin band, if not too dense, may occasionally be missed if it coincides with one of the main protein areas; if this is suspected—for instance on clinical grounds—the analysis should be made to include two-dimensional immuno-electrophoresis. The reverse error, of misidentifying a discrete beta or gamma band as an

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Fig. 5. Electrophoreses in agar gel (pH 8.6) of cerebrospinal fluid, concentrated 200 times, from a patient with multiple sclerosis (MS) compared to fluid concentrated 200 times from a normal person (NI) (by courtesy of Dr. E. Laterre)

Fig. 6. Selective rise of IgA concentrations in a patient who developed acute urinary infection after hip joint surgery

Concentrations of immunoglobulins are expressed as percentages of the normal mean

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immunocytoma product, is not at all impossible. Faint false "monoclonal" bands may be mimicked by the already-mentioned C-reactive protein, by hemoglobin from a hemolyzed blood sample (producing an adventitious band in the beta-1 zone), or even by fibrinogen or its breakdown products if plasma is inadvertently used instead of serum or if the serum was obtained from a patient with spontaneous or therapeutically induced clotting defects (Figure 7).

The distinction between genuine tumor products and homogeneous antibodies, which may have to be made when one or more faint bands are present, is usually a matter of clinical judgment that cannot be solved by the analyst. There are, however, some definite contributions that the analyst can make with regard to the question of whether a monoclonal immunoglobulin-producing disease is malignant or benign.

First, the presence of Bence Jones protein in the urine is nearly always diagnostic of malignant immunocytoma, as it is both frequent in this condition and exceptional in benign monoclonal disorders (28).

Second, malignant monoclonal proliferations are usually associated with a deficient production of all the other immunoglobulin classes, including that to which the abnormally increased monoclonal component belongs. Since this is not seen in benign conditions, the routine determination of IgG, IgA, and IgM should provide important information on this point.

Third, repeated measurements of the concentration of the monoclonal component, as described in the technical section, are apt to provide very precise information on the rate of growth of the tumor clone (29). To evaluate this information, one must take into account that the exchangeable pool of any protein, \( P \), and hence its concentration in the serum, is determined by the ratio of its rate of biosynthesis, \( s \), and hence the number of cells synthesizing it, to its fractional rate of catabolism, \( k \):

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P = \frac{s}{k}
\]

Because the fractional turnover rate is much higher for IgE (about 0.9 of the plasma pool per day) (17) than for IgA (0.25) or IgM (0.11-0.19), and particularly for IgG (about 0.07) (17), the tumor masses corresponding to a given concentration of these monoclonal components in the serum are also much greater in the case of IgE myeloma than with malignancies producing IgA, IgM or, particularly, IgG. Moreover, it is known that the fractional turnover rate of IgG increases with the concentration of this protein in the plasma, so that the line relating tumor mass and serum concentration in the case of IgG myeloma rapidly curves upward (Figure 8).

After a monoclonal immunoglobulin disorder has been picked up by the all-purpose serum electrophoresis test, it will be a simple matter to classify it immunologically by means of immunoelectrophoresis with use of specific antisera to heavy-chain classes and, what is less important, light-chain types. With more than 10 mg/ml of the monoclonal component being present, the heavy-chain diagnosis will already have been made by the associated routine IgG, IgA, and IgM determination. IgA proteins have a tendency to produce very broad bands on electrophoresis (Figure 7), and both IgM and IgA are only rarely found in the slow-gamma area.

Sometimes several monoclonal components are present; some of these may be serum Bence Jones proteins, but occasionally tumor products belonging to different heavy-chain classes or to members of different mobility within the same class may coexist in the serum. These are of considerable theoretical interest, but their discussion falls outside the scope of this paper. The same is true for the exceptional cases

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**Fig. 7.** Electrophoresis in agarose gel (pH 8.6) of sera from: N: a normal control; G: a patient with IgG myeloma; A: a patient with IgA myeloma (note typical diffuse appearance and fast mobility of the monoclonal component); L: a patient with myeloma producing only L-chains (serum Bence Jones protein); F: a patient under anticoagulant therapy (note presence of a fibrinogen band in the \( \beta_2 \) area); B: a patient with scleromyxedema of the skin and a long-standing benign immunoglobulin disorder producing minute amounts of a monoclonal IgG component.

**Fig. 8.** Relationships between the concentrations of IgE, IgA, IgM and IgG in the serum and the rate of biosynthesis of these proteins.

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in which incomplete molecules are synthesized, consisting only of a portion of the heavy chain; these should not escape detection if monoclonal components are systematically screened with a suitable battery of anti-heavy- and anti-light-chain antisera. The clinician cannot expect of the analyst that the latter also undertake a systematic search for antibody activity in the monoclonal components coming to his attention. It is suggested, though, that every serum of this kind be tested for precipitability in the cold, after twofold dilution with saline, since certain immune complexes produced by monoclonal immunoglobulins with rheumatoid factor (30) or antilipoprotein activity (31) may betray their presence by this simple test.

References