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## REVIEW

# LABORATORY TECHNIQUES OF VETERINARY CLINICAL IMMUNOLOGY: A REVIEW

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**Abstract**—Laboratory tests currently used or easily applicable in veterinary clinical immunology were reviewed in the following three categories: (1) tests detecting disorders in humoral immune response: serum protein screening tests, gammaglobulin estimation and complement testing; (2) tests detecting disorders in cellular immune response: phagocyte function tests and lymphocyte function tests; (3) tests detecting autoimmune disorders: detection of antinuclear antibodies, rheumatoid factor, autoimmune hemolytic diseases, and autoimmune organ and tissue disorders. The principles of the tests and the interpretations of the results are presented with the appropriate references.

**Key words:** Veterinary clinical immunology, humoral immune response, cellular immune response, autoimmune disorders, serology

## UNE REVUE DES TECHNIQUES DE LABORATOIRE UTILISÉE EN IMMUNOLOGIE CLINIQUE VÉTÉRINAIRE

**Resumé**—Les tests couramment utilisés et aisément applicables en immunologie clinique vétérinaire sont divisés en trois catégories: (1) Les tests détectant les désordres de la réponse immunitaire humorale: screening des protéines sériques, une estimation des gammaglobulines et des tests portant sur le complément. (2) Les tests détectant des désordres de la réponse immunitaire cellulaire: tests de la fonction de phagocytose et des fonctions lymphocytaires. (3) Les tests détectant des désordres autoimmuns: détection des anticorps antinucléaires, des facteurs rhumatoïdes, des maladies hémolytiques autoimmunes et des organes et tissus autoimmuns. Le principe et l'interprétation des tests sont présentés avec les références appropriées.

**Mots-clés:** Immunologie clinique vétérinaire, réponse immunitaire humorale: cellulaire, maladies autoimmunes, serologie

## INTRODUCTION

Clinical immunology has progressed in both human and veterinary medicine at a fast rate in recent years. Development or improvement of various laboratory techniques enabled the clinicians to diagnose immunologic diseases, for instance, immunodeficiencies and autoimmune disorders, which could be misdiagnosed without appropriate laboratory tests. There are two groups of laboratory immunologic tests currently used in medicine.

*Serologic tests* were developed after the discovery that antibody is capable of specific reaction with an antigen. One of the first reviews on serum reactions was published in 1917 [1]. Serologic tests are used successfully for over three-quarters of a century to *detect the presence of antibody* in the serum or other body fluids, or *the presence of the antigens* (bacteria, viruses, etc.) in various biological or laboratory materials. These techniques are described and reviewed in numerous textbooks of microbiology and mycology, and only those will be discussed here, which detect antibody to "self" antigens and are used in the diagnosis of autoimmune diseases.

*Clinical immunology laboratory tests* which are used in the evaluation of immunologic competence and autoimmunity are much younger. Their purpose is to detect the *ability of the host* (man, animal) *to respond* to the antigenic stimulation regardless of its source, or to *determine the dysfunctions* in the animals' immune system. There are numerous tests used in this category and those currently applied in veterinary clinical immunology will be discussed here.

There are five large groups of immunologically mediated disorders recognized in clinical patients: (1) defects of humoral immune response and factors; (2) defects of cell-mediated immune response and various cellular elements; (3) combined immunodeficiencies (4) autoimmunities; (5) allergies.

#### DETECTION OR DISORDERS IN HUMORAL IMMUNE RESPONSE

There are three large groups of humoral factors involved in immune reactions: (a) immunoglobulins; (b) non-specific serum factors, (e.g. complement, conglutinin); and (c) intercellular "messengers" (e.g. lymphokines, monokines).

##### *Immunoglobulins*

Immunoglobulins are the best known and studied immune humoral factors because of their relative stability and specificity. The basic features of immunoglobulins in various species were reviewed in a textbook [2]. Whereas in serology the antibody activity of the immunoglobulins is studied with regards to its specific action with the given antigen, in clinical immunology the ability of the host to produce immunoglobulins (regardless of their antibody specificity) is tested. Immunoglobulins, as any other serum proteins, are known to lead to antibody formation if injected in another species. Consequently, immunoprecipitation is still the most commonly used technique for detection and quantitation of immunoglobulins in the blood serum. The absence of detectable immunoglobulins in colostrum-fed newborn and in adult animals indicates various types of immunodeficiency, usually, but not necessarily, associated with dysfunctions of antibody producing cells of B cell origin. The excess of immunoglobulins indicates either infections or a cancerous disease of immunoglobulin producing cells known as myeloma.

##### *Non-specific serum factors*

There is a number of recognized non-specific serum factors of which only the proteins of the *complement system* are usually tested in connection with clinical cases. The complement system is a system of naturally occurring serum proteins which upon their activation act as enzymes for other complement components, or produce biologically active split factors, or have a "detergent-like" action on the cell membranes. The cell membrane damage and destruction of the cell or the microorganism, which cannot be achieved by antibody alone, is probably the most important biological function of the complement system [3]. Though we have only a limited knowledge about complement and complement component levels in domestic animals, various disorders were identified in man and laboratory animals as being associated with the complement system dysfunctions.

### *Intercellular "messengers"*

The third group of humoral factors, i.e. the intercellular messengers, is the most recently recognized group of humoral factors functioning as "activators", "stimulators", "inhibitors", etc., of the action of other cells. So far the best known are numerous *lymphokines* or *products of activated lymphocytes* which are being identified since 1964 [4] when the amplification of inflammatory reaction by sensitized lymphocytes was described. Similar products produced by monocytes and granulocytes are being identified or may be expected to be discovered. These products are usually studied in connection with the cell-mediated immunity and have no clinical diagnostic value known at the present time.

### SERUM PROTEIN SCREENING TESTS

To avoid complicated and expensive tests used for detection and quantitation of specific immunoglobulins some simple pre-tests are recommended.

#### *Total protein estimation*

*Refractometric estimation.* A simple refractometric estimation of serum proteins is fast and gives sufficiently accurate results for a screening test. Many types of a refractometer (protometer) may be used. The procedure for total protein estimation is supplied by the producer with the instrument. It is based on the study on refractive indices of protein solutions by Perlman and Longworth [5]. Many of the refractometers have directly engraved scales for total serum protein estimation. If only the refractive index is given, a nomogram may be used for the protein estimation (Fig. 1.) The protein concentration may be determined to the nearest 0.1 g %, which is sufficient for evaluations of most clinical cases.

*Biuret assay and Lowry method.* If more precise estimation is necessary, which happens seldom in clinical immunology, the Biuret assay may be used as described by Cornwall *et al.* [6]. The sensitivity range of the Biuret method is between 0.25 to 200 mg of protein per ml. A more sensitive method was described by Lowry *et al.* [7]. It is a modification of the Folin-Ciocalteu method and is based on the detection of tyrosine and tryptophane in

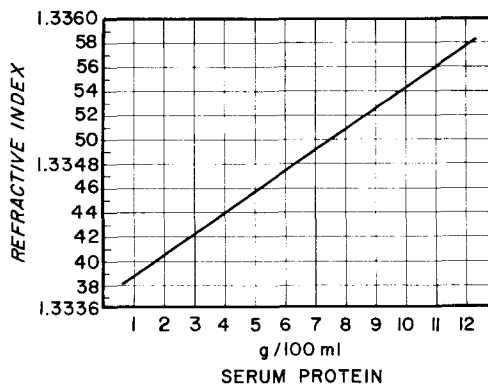


Fig. 1. Nomogram for determination of serum protein concentration from refractive indices

proteins. The sensitivity ranges between 0.001 and 0.2 mg of protein per ml of the sample.

*Ultraviolet absorption analysis.* This method was described by Warburg and Christian [8] and is used only with diluted serum protein samples. The amount of protein in mg per ml =  $1.55 \times OD_{280} - 0.775 \times OD_{260} \times \text{dilution factor}$ .  $OD_{280}$  and  $OD_{260}$  means the optical density readings at 280 and 260 nm wavelengths, respectively.

### *Interpretation of the results*

Normal values of plasma protein in healthy animals are given in the Table 1. Age is probably the most important factor which has to be taken into account when evaluating the results: newborn and very young animals may have values up to 60% lower, between 3

Table 1. Normal values of plasma protein in healthy animals (in g/100 ml) (Schalm *et al.* [9]).

| Cat     | Cow     | Dog     | Goat    | Horse   | Sheep   |
|---------|---------|---------|---------|---------|---------|
| 6.0-7.5 | 6.0-8.0 | 6.0-7.8 | 6.0-7.5 | 5.3-7.8 | 6.0-7.5 |

and 5 g/100 ml without being clinically ill. However, serum samples taken after colostrum feeding should be close to the minima indicated in Table 1. Adult animals usually show values close to the maximum given for normal animals. Additional variation may stem from the breed, sex, and individual differences.

*Hyperproteinemia* could be observed in: (1) various chronic infectious diseases [9]; (2) dehydration due to extreme heat and insufficient water supply or due to chronic nephritis [9]; (3) multiple myeloma [10].

*Hypoproteinemia* could be observed in: (1) newborns and colostrum-deprived animals [9]; (2) animals with hemorrhages of various origin [9]; (3) glomerulonephritis and renal amyloidosis [11]; (4) chronic liver diseases due to insufficient albumin synthesis [9]; (5) Johne's disease (paratuberculosis) due to the loss of albumin [9]; (6) protein losing enteropathy [12].

## GAMMAGLOBULIN ESTIMATION

Because the changes in antibody are reflected mainly in the gamma and beta 2-globulins of blood serum, their estimation gives an important information about the status of specific humoral immunity and indirectly about the functions of antibody producing B cells.

### *Electrophoresis*

Serum electrophoresis requires only microliters of serum samples and can assist in the diagnosis of many disorders. It gives a percentage distribution of serum albumin and globulins, and when multiplied by the total serum protein concentration, it gives their absolute concentration values. However, electrophoresis tends to underestimate extremely high concentrations of gammaglobulins [13] which is caused by an "overflow" of one globulin into the region of the other with poorly expressed division points between the

globulins. The optimal procedures for electrophoresis are given by the producers of the equipment used. Commonly sodium-barbital, Tris-barbital, or Tris-tricine buffers, pH 8.6–8.8 are used for serum electrophoresis. We use commercially prepared Tris-barbital-sodium barbital buffer of pH 8.8 and ionic strength 0.05 with very good results (Fig 2). The buffer composition is: Trizma (Tris) 5.077 g, barbital sodium salt 9.81 g, barbituric acid 2.45 g per 1000 ml of distilled H<sub>2</sub>O. Serum electrophoresis requires special equipment not always available to the clinicians. Thus, following simple techniques have been applied for detecting hyper- or hypogammaglobulinemias.

#### *Zinc sulfate turbidity*

The reaction is based on detecting of turbidity created by zinc sulfate solution added to the serum sample as described by McEwan *et al.* [14]. A solution of 208 mg of ZnSO<sub>4</sub> per 1 litre of distilled water deprived of CO<sub>2</sub> by boiling is used in the test, in which 6 ml of this solution is added to 0.1 ml of the test serum. After agitation and 1 hr incubation, the absorbance is read at 660 nm in a spectrophotometer. The absorbance reading is translated to milligrams of gammaglobulins per milliliter, using a standard curve prepared from serum dilutions of known gammaglobulin concentration. The results obtained by this method correspond well with the gammaglobulin concentration in the serum [13] in sera without hemolysis. The effect of hemoglobin coloring in the serum sample on the detected gammaglobulin level can be minimized by using a correction equation [13]:

$$\text{Actual gammaglobulin content} = \text{Apparent gammaglobulin content (mg/ml)} - (\text{Absorbance of 1:20 serum dilution at 540 nm} \times 23).$$

#### *Glutaraldehyde coagulation*

Glutaraldehyde in a low concentration will polymerize practically only gammaglobulins at pH 7.4. This property has been utilized in testing human [15], bovine [16], canine [17] and mink sera [18]. Either 6% (2.4 ml glutaraldehyde 25%, Merck No. 4239 + 7.6 ml distilled water) [17] or 10% solution (4 ml glutaraldehyde 25% + 6 ml distilled water) [16] were used for dog and calf gammaglobulin estimation, respectively. The concentration of gammaglobulin is proportional to the coagulations time (Fig. 3). Samples which do not coagulate within 1 hr are suggested hypogammaglobulinemic. Hypergammaglobulinemia is expressed by coagulation in less than 1.5 min. The gammaglobulin concentration may be estimated from the graphs presented in literature [18, 19].

#### *Radial immunodiffusion*

Radial immunodiffusion, also called Mancini's method [20], is a single diffusion procedure in which the serum sample being tested for presence of an immunoglobulin diffuses through an agarose gel containing specific antiserum against the given immunoglobulin. Commercially prepared plates with agarose containing specific antiserum to given immunoglobulins are available allowing to test the concentration of IgG in most species and IgM, IgA, IgG(Y) in some species. Each kit for detecting the immunoglobulin concentration is supplied with standards of known immunoglobulin concentration and procedure description. The precipitation ring radius developed by the given serum sample (Fig. 4) in a given time is compared with the radii obtained with the standards and plotted

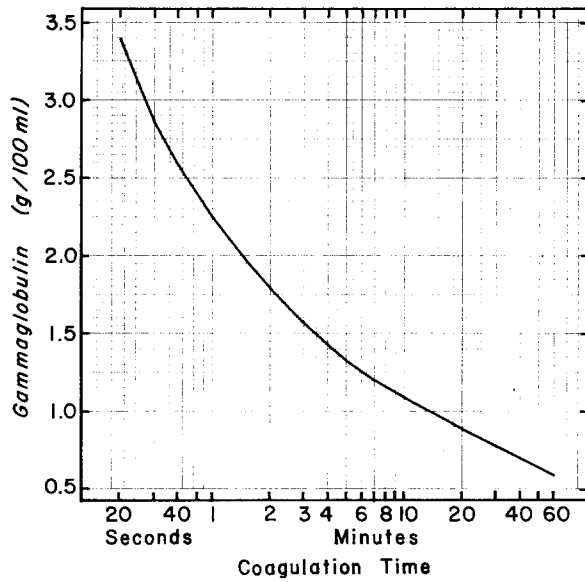


Fig. 3. Nomogram expressing relation of the glutaraldehyde induced coagulation time to the gammaglobulin concentrations in the serum. Based on data published in [19].

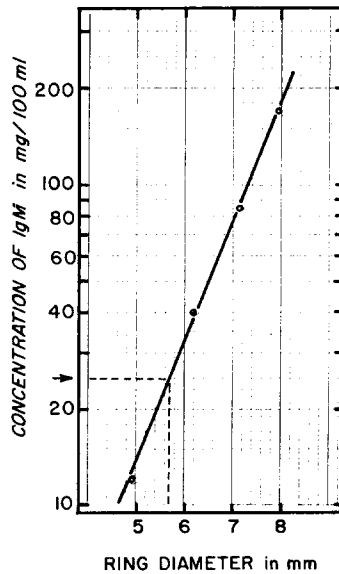


Fig. 5. A nomogram for the determination of equine IgM from an actual radial immunodiffusion plate measurements with standards of 12, 40, 85 and 170 mg IgM/100 ml serum and a foal serum sample (broken lines) with 25 mg IgM/100 ml serum.

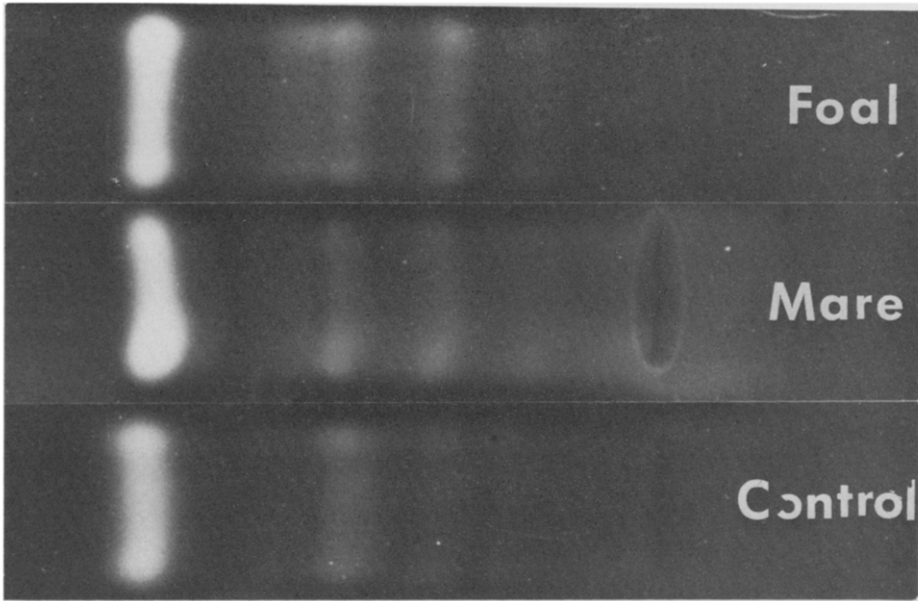
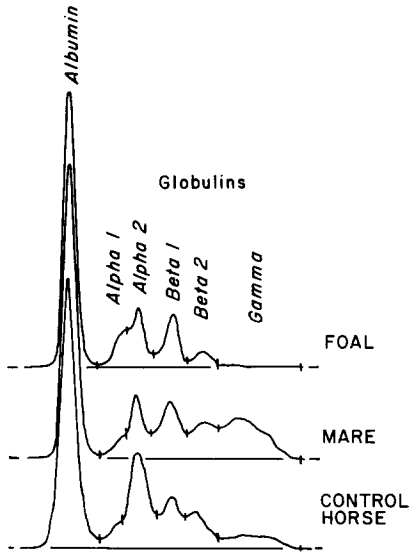


Fig. 2. Electrophoretic patterns of a hypogammaglobulinemic serum from a foal, hypergammaglobulinemic serum from a mare, and a serum sample from a normal healthy horse. 2A – scanning curves of the electrophoretogram, 2B – black and white print from acetate plates stained with Ponceau's protein stain.

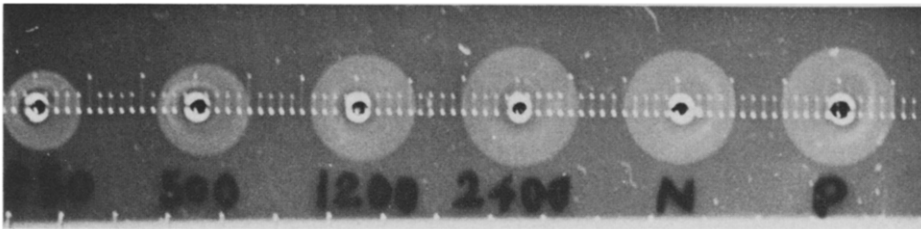


Fig 4. Radial immunodiffusion determination of canine IgG in four standards containing (from left to right) 250, 500, 1200 and 2400 mg IgG/100 ml serum, respectively, and in two sera "N" and "P".



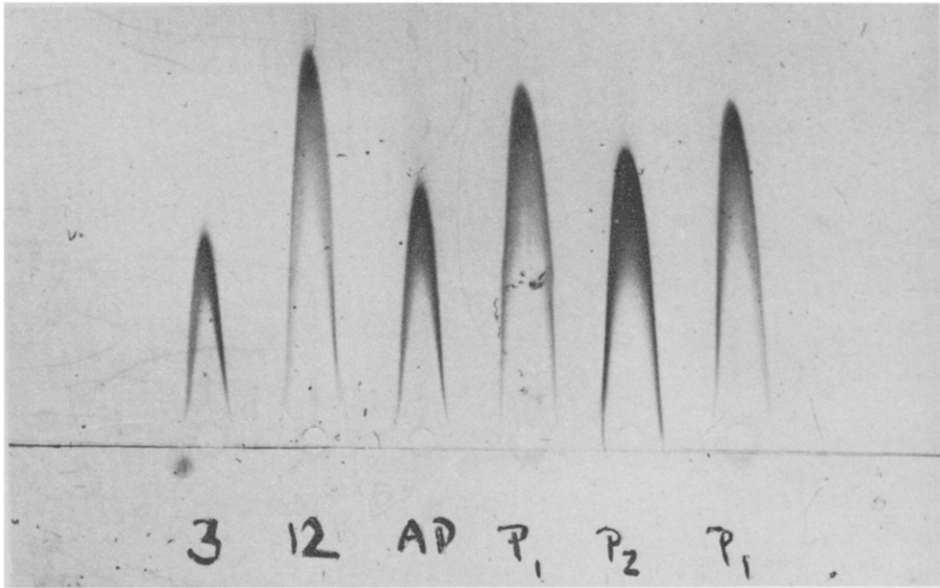


Fig. 6. A rocket immunoelectrophoretic pattern produced by two standards of 30 and 120 mg of canine IgA/100 ml serum and by three patient samples AP, P1 and P2 containing 40, 98 and 89 mg IgA/100 ml serum, respectively. The calculation was done on the area basis of the precipitates.

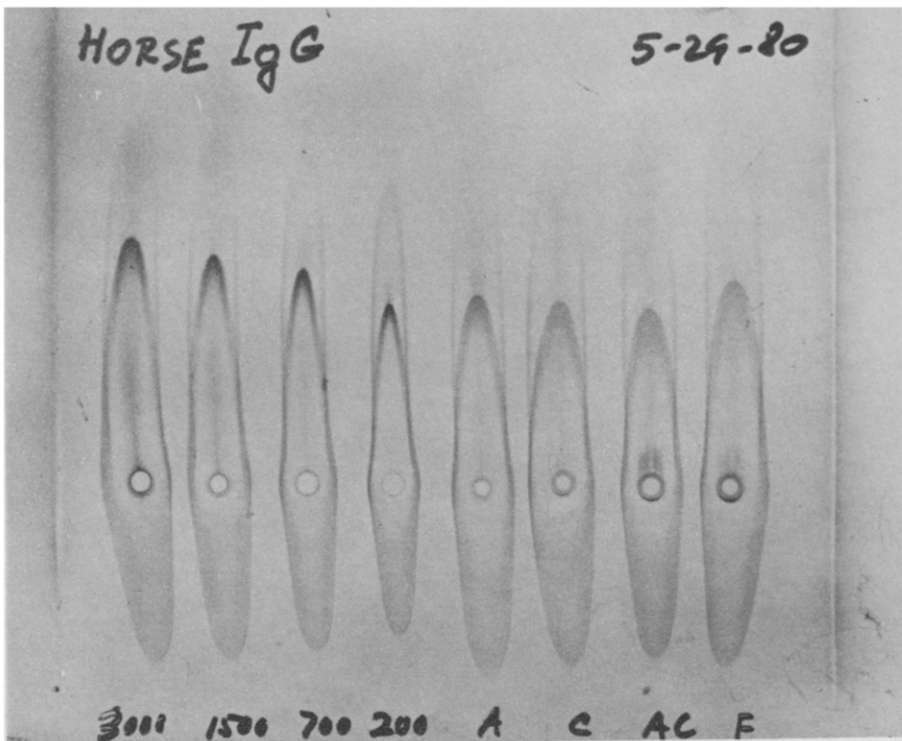


Fig. 7. Rocket immunoelectrophoresis of equine IgG. The antiserum used had antibodies to both a slow IgG (forming a "cigar" shaped precipitating line) and the fast IgG (forming a lighter rocket shaped precipitating line). The quantitation of the slow IgG was possible by comparing the area of the "cigars" formed by the standards (from 3000 through 200 mg IgG/100 ml serum) with the areas formed by the patients' samples marked A, C, AC and F and containing 1600, 2150, 1180 and 2000 mg IgG/ 100 ml of serum.

on semilogarithmic paper (Fig 5). The diagnostic plates for radial immunodiffusion may also be prepared in the laboratory [20, 21]. It has to be stressed that high specificity antisera have to be used for reproducible results. The technique is relatively accurate, but may give lower than actual reading when higher concentrations of immunoglobulin are present in the serum samples.

#### *Rocket immunoelectrophoresis (Laurell)*

This relatively new technique was introduced in 1966 by Laurell [22]. It was primarily used to measure strongly negatively charged proteins as is albumin, but may be used for other proteins as well. The method is described in detail in the original Laurell's paper [22] and in a manual [23]. We have used this method for quantitation of immunoglobulins utilizing specific commercially prepared antisera against the given immunoglobulins. In principle, the antigen, e.g. the tested serum (prediluted to 1:10 or 1:20) is placed in constant volumes (5  $\mu$ l) in the wells (2.5 mm diameter) in a layer of agarose containing 1/2 to 1% concentration of specific antiserum against the given immunoglobulin in question. The plates are then subject to electrophoresis (7–8 V/cm for 3 to 5 hr) during which a precipitating line develops in the region of optimal proportions of the antigen and antibody. After electrophoresis, the plates are washed and stained in a similar way as other immunoelectrophoretic slides. After washing out the unprecipitated protein, the precipitates are stained. Figure 6 illustrates "rockets" of IgA from dog serum. Because IgG migrates to both poles from the well, the well has to be located close to the center of the agarose plate and the resulting precipitating line has a cigar shape (Fig. 7). The multiple of the length times average width of the "rocket" or "cigar" is directly proportional to the concentration of the immunoglobulin in the serum. We have successfully used both the original barbital-sodium barbital-calcium lactate buffer (pH 8.6, ionic strength 0.015 for agarose and 0.025 for vessels) [23] and Tris-tricine-calcium lactate buffer (pH 8.6: 0.08 M Tris, 0.024 M Tricine, 0.3 mM calcium lactate, and 0.02% sodium azide) marketed as QIEP IV buffer (Bio-Rad, Richmond, CA). A set of controls with known immunoglobulin concentrations is included with each set of samples. The immunoglobulin estimation may be completed in one day. The accuracy is comparable to or better than in radial immunodiffusion.

#### *Crossed immunoelectrophoresis (Laurell)*

Crossed immunoelectrophoresis is a combination of two electrophoretic separations: one of the proteins in agarose gel without antibody followed by electrophoresis perpendicular to the first one into antibody-containing gel. The resulting pattern (Fig. 8) is composed of peaks of overlapping precipitating lines. The area enclosed in an individual precipitate is proportional to the antigen/antibody ratio of the system and, therefore, planimetric measurement may quantitate the concentration of the given protein. The procedure was described in detail in the review manual [23].

By utilizing specific anti-immunoglobulin serum in the agarose for the second dimension electrophoresis, an estimation of the specific immunoglobulin content is possible. The antiserum used in the case illustrated in Fig. 9 detected 3 subclasses of cat IgG. The cases of hyper- or hypogammaglobulinemia, which cannot be easily detected by normal immunoelectrophoresis, are well noticeable in crossed immunoelectrophoresis.

### *Interpretation of results*

Hypergammaglobulinemia is usually suggested when the gammaglobulin exceeds 2.0 g/100 ml serum and hypogammaglobulinemia when it is below 0.5 g/100 ml serum. However, these values have to be applied cautiously.

Natural hypogammaglobulinemia appears in newborns of all domestic animals [24]. In absolute values, even postcolostral sera may be at the low "normal" level of gammaglobulin. Decreased gammaglobulin levels were noticed in many physiologic and pathologic conditions. In cows, for instance, decrease of beta 2- and gamma globulins was noticed before parturition [25]. Also, larger blood loss will lead to lower absolute values of gammaglobulins. Hypogammaglobulinemia appears in many cases of immunodeficiencies, for instance, in Arabian horses [26], in colostrum deprived animals or animals with failure of absorption of colostrum immunoglobulins [27].

On the other hand, it has to be emphasized that some young immunodeficient animals need not be hypogammaglobulinemic, if they absorb a high level of colostrum gammaglobulins. Because the IgM class of immunoglobulins has substantially shorter half-life than IgG class (approximately by 2/3), and because it is absorbed in smaller amounts from the colostrum, colostrum IgM disappears from the blood circulation sooner. Its sub-normal levels are used as an indicator of immunodeficiency in young animals, in which the levels of circulating IgG may be normal due to the persistence of absorbed colostrum IgG. Thus, these IgM deficiencies may be detected by the last three mentioned tests; i.e. radial immunodiffusion, rocket or crossed immunoelectrophoresis, but not by zinc sulfate precipitation or glutaraldehyde coagulation.

Most infectious diseases, chronic infections in particular, will lead to hypergammaglobulinemias [28] (Fig. 8). Very high levels of gammaglobulins (over 25% of total protein) are, for instance, present in cattle naturally infected with foot-and-mouth disease virus [29], in cats with feline infectious peritonitis [30], in minks with Aleutian disease [31,32] and other infections. An extraordinary increase in beta 2- and gammaglobulins (up to over 50% of total serum protein) is found in cases of tumors of lymphoid cells causing macroglobulinemia [33] or multiple myeloma [34], respectively.

For a veterinary clinician, hypogammaglobulinemia in colostrum-fed young animals and hypergammaglobulinemia in animals with no apparent infectious diseases should be of major concern. Hypogammaglobulinemia in young animals may indicate immunodeficiency, or failure of passive transfer of colostrum antibodies. Hypergammaglobulinemia without concurrent infection may indicate myeloma or autoimmune disorders. All of the above mentioned conditions have guarded on poor prognosis. On the other hand, hypergammaglobulinemia associated with infectious diseases is an important defense feature and will disappear when the infectious agent is eliminated, thus it is a favorable feature. The immunoglobulin concentrations in the serum, colostrum and milk were summarized recently [35]. In general, there are variations in the reported level of immunoglobulins due to the species and age, but also due to the variation of techniques and the quality of the reference standards. IgG shows the highest immunoglobulin concentration in the serum and has 2 to 4 subclasses in various species. The reported levels of IgG in clinically healthy horses and dogs ranges from around 500 to over 1700 mg/100 ml, whereas that for the ruminants and pig generally exceeds 1700 up to over 2500 mg/100 ml. On the other hand chicken has only 300 to 700 mg/ml reported.

IgM levels are higher than IgA levels in ruminants (150 up to 400 mg/100 ml) and

chicken (230–250 mg/ml), slightly higher in the dog (120–250 mg/100 ml), and about the same as IgA levels in the horse (80–200 mg/100ml) and pig (100–500 ml).

IgA levels are the lowest in ruminants (10–50 mg/100 ml) and chicken (30–60 mg/100 ml) and slightly higher in the pig and horse (50–500 mg/100 ml) [35].

## COMPLEMENT TESTING

The complement system is a very important system of serum proteins which augment the immune reactions in the body. The complement components become activated by various ways, most commonly by antigen–antibody complexes [36]. Consequently, the decreased level of complement usually reflects an increased formation of immune complexes. The proteins of the complement system are produced by the animal body naturally and are kept at “physiological” levels as much as possible. There are known deficiencies of complement components or their inhibitors in man [37] and inbred animals [38] and dozens of new cases are reported every year. Testing of levels of total complement activity in serum, single complement components, or inhibitors assists in diagnosis and prognosis.

### *Total hemolytic complement determination*

The determination of total complement activity in domestic animals is complicated by the variation in optimal target red blood cells for the complement system of a given species and by variations in optimal test conditions. In addition to this, the complement system is composed of a number of components, some of which are heat labile and the resulting titer reflects both the original levels of complement in the animals and the conditions of handling and storage of the blood and serum sample prior to testing. Thus, in order to obtain reproducible results, the blood handling and storage and the testing conditions must be scrupulously standardized. A control sample tested earlier must be included with each set of tests. We recommend using 45–60 min room temperature clotting time, ice water bath temperature after that time, and harvesting the serum sample no later than 1.5 to 2 hr after blood collection. If a secondary “white” clot appears in the serum after storage, the resulting titer is decreased. Samples of horse and sometimes cattle sera are most commonly forming secondary clots. The harvested serum should be tested immediately or quickly frozen on dry ice-methanol or ethanol bath and stored at  $-70^{\circ}\text{C}$ . With the noticeable exception of swine serum, other species complement levels did not change in our laboratory at  $-70^{\circ}\text{C}$  storage for several months. Swine serum should be tested immediately. Standard control samples of swine sera slowly decayed in complement activity even at  $-70^{\circ}\text{C}$ .

The optimal conditions for testing hemolytic complement in various species of domestic animals are summarized from the literature [39–46] in Table 2. It may be emphasized that other than conditions listed in Table 2 will also lead to the detection of hemolytic complement activity, usually at lower titers. The most important factors in complement level determinations are: (1) identical handling and storage of the serum samples tested; (2) using of constant concentrations of sensitized red blood cells; we recommend  $1 \times 10^8$  cells/ml determined by optical density measurement at 412 nm; (3) using of optimal concentration of sensitizing antibodies against the given red blood cells; we recommend a dose which will give the highest detectable titer of complement, but which will not

Table 2. Optimal target cells, buffer and testing conditions for testing hemolytic complement in the serum of domestic animals

| Source of complement | The most sensitive target erythrocytes | pH   | Ionic strength | Optimal buffer concentration of Mg <sup>2+</sup> + Ca <sup>2+</sup> in millimoles | Optimal temperature (°C) | CH50 titers in healthy adults | Reference |
|----------------------|----------------------------------------|------|----------------|-----------------------------------------------------------------------------------|--------------------------|-------------------------------|-----------|
| Bovine               | Guinea pig                             | 7    | 0.09           | 1                                                                                 | 37                       | 80-450                        | 39,40     |
| Canine               | Sheep                                  | 7.3† | 0.075          | 1                                                                                 | 37                       | 120-320                       | 41        |
| Caprine              | Guinea pig                             | 8.0  | 0.09-0.12      | 1                                                                                 | 37                       | 18-75                         | 42        |
| Equine               | Swine                                  | 8.0‡ | 0.12           | 1                                                                                 | 37                       | 10-24                         | 43        |
| Feline               | Guinea pig                             | 7.3  | 0.06           | 1                                                                                 | 37                       | 70-150                        | 44        |
| Ovine                | Rabbit                                 | 6.5  | 0.12           | 1                                                                                 | 37                       | 30-64                         | 45        |
| Porcine              | Sheep                                  | 7.3  | 0.09           | 1                                                                                 | 37                       | 75-210                        | 42        |
| Avian (Chicken)      | Swine                                  | 8.0  | 0.15           | 1                                                                                 | 30                       | 30-80                         | 46        |

\* The standard isotonic NaCl-barbital-sodium barbital buffer (Kabat, Mayer) (BB) is listed as ionic strength of 0.15. The other ionic strength buffer are prepared by mixing this with 5.0% of glucose (G) or sucrose solution, which has practically no ionic strength: 0.12=4 parts BB+1 part G, 0.09=3 parts BB+2 parts G, 0.075=1 part BB+1 part G.

† We have found pH 7.3 to be superior when buffer of ionic strength of 0.075 is used, whereas pH 6.5 given in [41] was superior when buffer of ionic strength 0.15 was used.

‡ Higher pH than 8 often causes nonspecific lysis: therefore we recommend this pH instead of pH 9 reported optimal in [43].

agglutinate the red blood cells; (4) using of standard pH and conductivity conditions of the buffer — see Table 2; (5) using of standard temperature and timing conditions of the test; we recommend a temperature of 37°C for mammalian complements and 30°C for chicken complement with a determination of lysis after 90 min. Both tube and microtiter plate techniques may be used. The determination of the degree of lysis is done after centrifuging the cells down in both tube and microtiter plate determination. A skilled laboratory worker may determine the degree of lysis to  $\pm 5\%$  just from the size of sedimented cell volume and the color of the supernatant fluid, which is sufficient for clinical testing. More accurate measurement may be done using a spectrophotometer at 412 nm, which is the peak of absorbance for hemoglobin in aged cells.

#### *Testing of complement components*

Functional testing of complement component levels is done by supplying 100-fold excess of components not in question. Commercial preparations of sensitized sheep erythrocytes treated with antibodies and early acting components of the complement system and purified components isolated from guinea pig or human sera are available and may be used in testing complement components in a number of species of domestic animals [47]. Only C2, C4 and C3 from herbivorous animals cannot be tested by the available system and no simple or commercially prepared testing system has been published for these components.

Immunochemical quantitations of C3 and some other components may be performed by rocket immunoelectrophoresis with specific antisera to them. These procedures detect the presence of the given complement component regardless of its function. They are used in human but not yet in veterinary medicine at the present time.

#### *Interpretation of results*

The hemolytic complement levels in CH50 units per ml of serum in clinically healthy adult animals are summarized in Table 2. Horse serum has the lowest detectable level of hemolytic complement even under optimal conditions, probably due to a quick conversion of the C3b into C3d sites, which favors more conglutination than lysis of red blood cells. Therefore the conglutinating complement titers in horse serum exceed considerably the hemolytic titers. Bovine or other ruminant sera have a similar tendency when tested with sheep erythrocytes, but not when tested with guinea pig or rabbit erythrocytes. Horse, goat, sheep and chicken sera give low hemolytic titers, though they are highly active in complement mediated bactericidal activity [48, 49].

Only results of testing done under completely identical serum handling and test conditions may be compared with each other. The best control is to run a standard serum reference sample kept frozen at  $-70^{\circ}\text{C}$  or lower temperature in small aliquots simultaneously with each series of sera from clinical patients. The titers will be usually slightly lower with samples frozen and once defrosted than in fresh samples. Twice frozen and defrosted samples should not be used for testing.

Hypocomplementemia is suggested in samples in which the CH50 units are 50% or less of the lowest level detected in clinically healthy animals. Physiological hypocomplementemia is present in newborn animals [44, 50]. Their level of complement reaches the level of adult animals within weeks to months. Pathological

hypocomplementemia may be caused by infections, automimmune diseases or inherited defects in complement component production or function [37, 51]. The clinical meaning of complement levels higher than in "normal" healthy animals is not clear.

Testing of individual components of the complement system has not been done in clinical patients in veterinary medicine and no interpretation is available. The values detected in clinically healthy animals were published [47]. The defects of individual components production or function in man either caused no clinical symptoms or were associated with various infections [37, 51].

#### INTERCELLULAR "MESSENGERS"

Determination of intercellular "messengers" such as lymphokines and monokines is used in research, but has not been applied in clinical immunology so far. Indirectly the production of these humoral factors is tested by various tests of cell mediated immunity. It is premature to review this topic in connection with veterinary clinical immunology.

#### DETECTION OF DISORDERS IN CELLULAR IMMUNE RESPONSE

The functions of two groups of cells are tested in clinical patients: (a) phagocytes and (b) lymphocytes.

##### *White blood cell count*

The total white blood cell count and a differential count are often indicative of disorders in cellular immunity [9]. Lymphopenia may be associated with defects of T or T and null cells primarily. Neutropenia is often associated with increased susceptibility to infections, with so-called "lazy leukocyte syndrome" and T cell deficiencies [52]. The increased number of immature forms of white blood cells indicates their heavy use or is associated with leukocytosis in numerous tumorous diseases [52].

#### PHAGOCYTE FUNCTIONS

The tests for phagocyte functions are generally still in their development even in human medicine. At the present time, a number of defects can be uncovered. Whether they appropriately explain the symptomatology or measure the *in vivo* processes is open to question [52]. The problems of neutrophil function tests were discussed recently [53]. Very few tests of phagocyte functions were used so far in veterinary clinical immunology [54].

##### *Epinephrine stimulation*

Mobilization of neutrophils from the body depot is tested by subcutaneous injections of epinephrine (1:1000). The capillary blood samples are taken before and up to 60 min after the injection. There should be an increase of peripheral polymorphonuclears of over 45% detected within 1 hr [55].

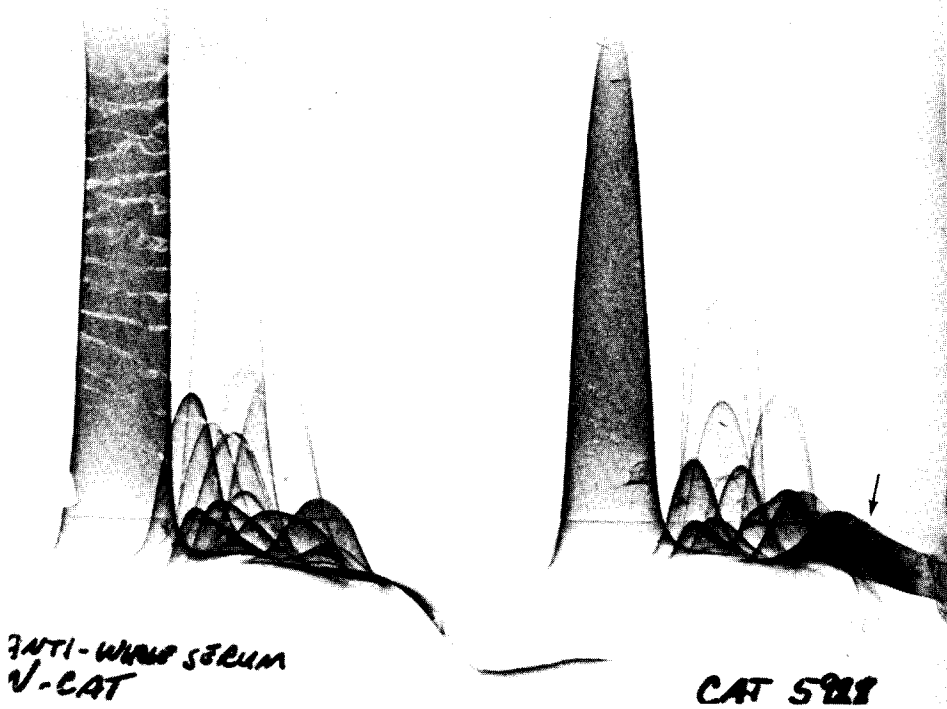


Fig. 8. Crossed (two-dimensional) immunoelectrophoresis of cat serum shows hypergammaglobulinemia due to the increase in IgG in the patient's serum. The precipitation pattern was developed with an antiserum to whole cat serum.

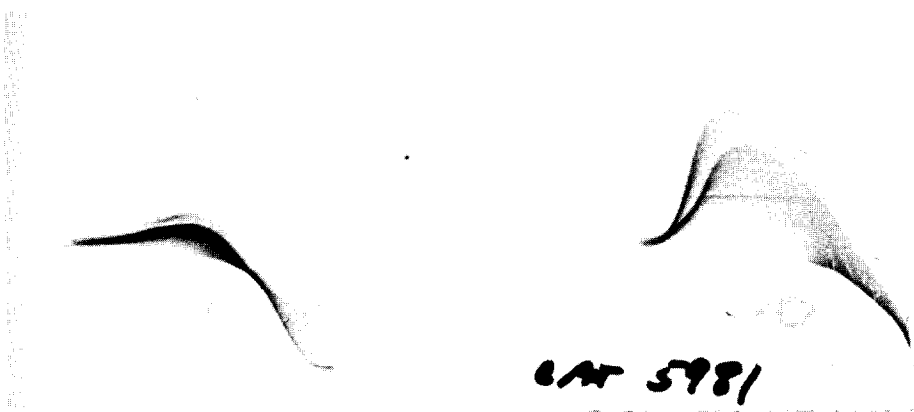


Fig. 9. Crossed immunoelectrophoresis utilizing specific anti-cat IgG antiserum indicated that the hypergammaglobulinemia in the patient's serum used in Fig. 8 was due to the elevation of two subclasses of IgG.





Fig. 10. Immunofluorescence of VERO tissue culture cell nuclei due to antinuclear antibodies in two canine patients. (a) Peripheral staining typical for acute cases with speckled staining in the nucleus often present in progressive systemic sclerosis. (b) Diffuse staining, which is less frequently detected and which is usually associated with chronic autoimmune diseases of systemic lupus erythematosus type.

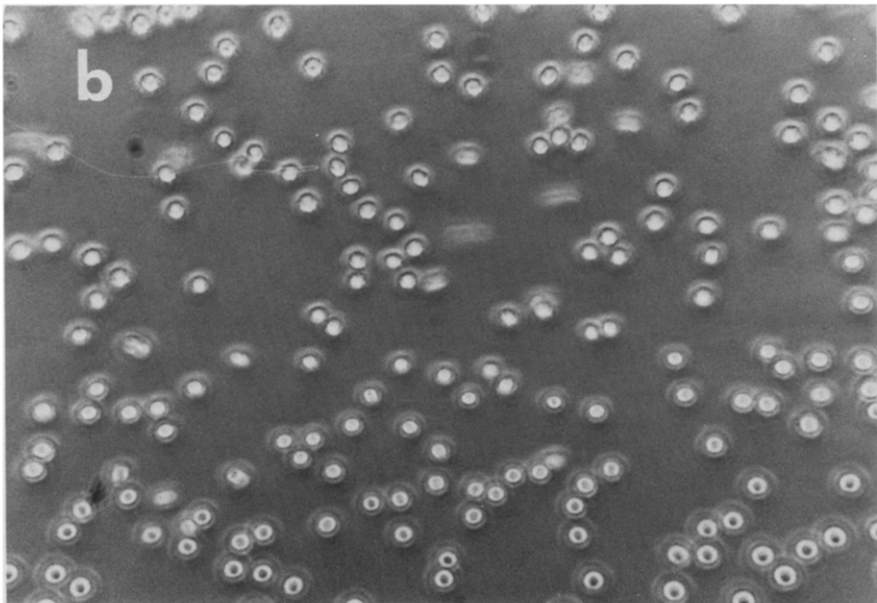
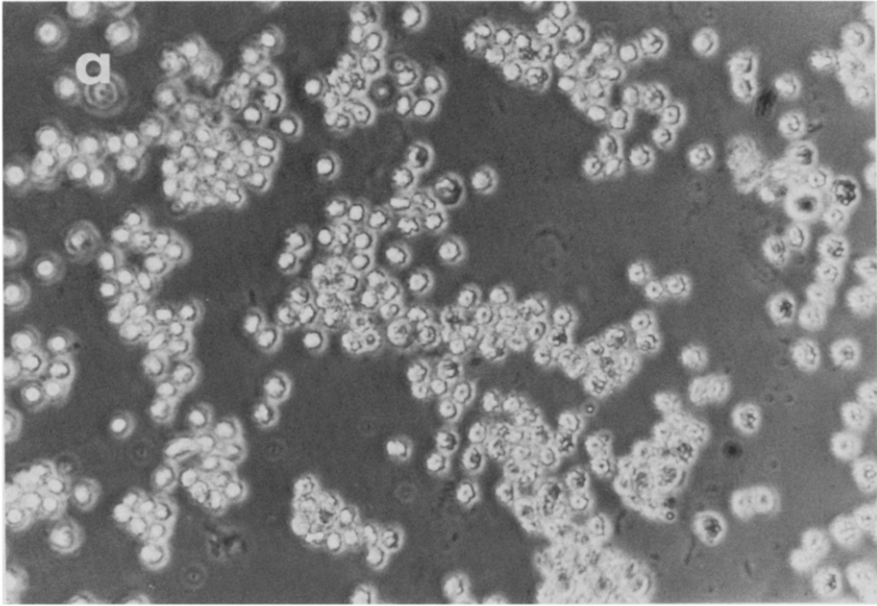


Fig. 11. Positive (a) and negative (b) results of the antiglobulin (Coombs) test observed under a phase contrast microscope. Similar observation may be obtained in Rose-Waaler tests for rheumatoid arthritis.

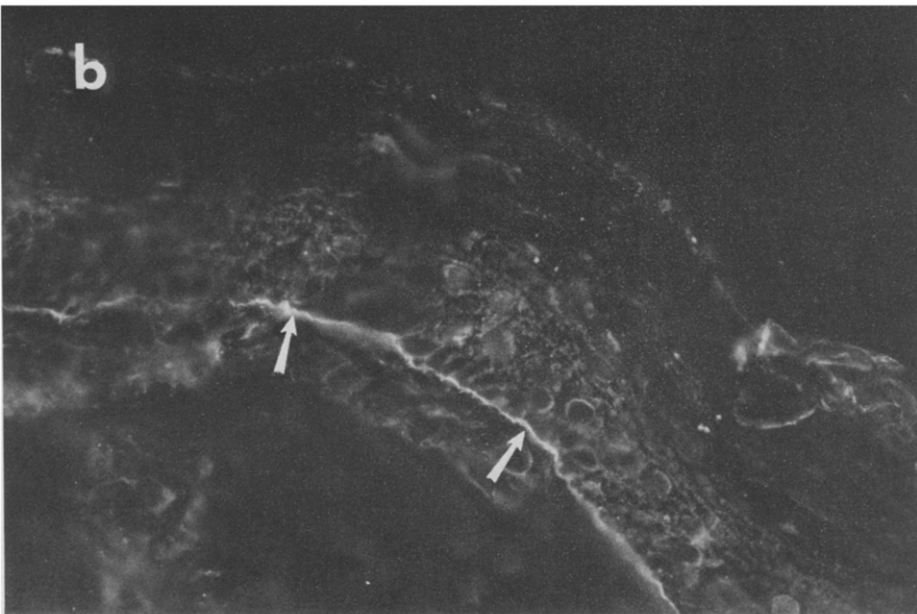


Fig. 12. A direct immunofluorescence technique (a) detected IgG deposits at the skin basement membrane from a dog with bullous pemphigoid disease. The same dog had in its serum circulating antibody which deposited on the basement membrane of healthy dog skin section as detected by an indirect immunofluorescence technique (b).

### *Nitroblue tetrazolium ingestion and digestion tests*

Nitroblue tetrazolium is directly reduced by superoxide anion to an insoluble purple formazan after phagocytosis by an active polymorphonuclear cell. Therefore its reduction is a measure of superoxide anion generation by the polymorphonuclears. Two modifications were applied in human medicine: one testing the polymorphonuclear oxidative functions *in vitro* followed by spectrophotometric measurement [56] and the other by staining a blood smear and counting the formazan-containing cells in a blood smear [57]. In veterinary medicine the test was used experimentally [54] and we are starting to use it in testing clinical cases.

### *Determination of iodination*

This test was recently used successfully in the study on bovine polymorphonuclear cell function [54]. The iodination test measures the ability of the polymorphonuclears to convert inorganic iodine to a trichloroacetic acid precipitable form, i.e., protein-bound. The iodine is covalently bound to a suitable acceptor molecule, such as the tyrosine residues of proteins via the actions of hydrogen peroxide and myeloperoxidase. This system has been found to exhibit a marked toxic activity toward bacteria, fungi, and viruses [58, 59]. The iodination procedure was recently re-evaluated with human polymorphonuclears [60]. The procedure is based on determination of radiolabeled iodine bound to the polymorphonuclears after incubation of the cells with preopsonized zymosan, NaI, and  $^{125}\text{I}$ .

### *Bactericidal killing and biochemical assessment*

Various modifications of bactericidal tests utilizing *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* or other bacteria were used [54]. The tests are quite laborious and the results are affected by numerous factors. Consequently, they were not tested sufficiently in clinical patients of domestic animals. Similarly, an extensive biochemical analysis of antimicrobial mechanisms of the neutrophils was not utilized in veterinary clinical immunology.

### *Interpretation of results*

Epinephrine stimulation fails to induce a 45% or greater increase in peripheral blood neutrophils in patients with "lazy leukocyte syndrome" [61] described only in man. Nitroblue tetrazolium is oxidized to formazan in more than 30% neutrophils in healthy individuals but decreased in children with chronic granulomatous disease (CHGD) [57]. The digestion/ingestion (D/I) index for nitroblue tetrazolium is over 2.5 in normal healthy man and was in our laboratory with bovine and canine polymorphonuclears. A decreased D/I index was detected in children with CHGD [56] and in dogs with severe chronic skin infections tested in our laboratory. These dogs might have had a defect similar to CHGD prior to their skin conditions. The preliminary results of phagocyte testing in domestic animals are promising [54] and may lead to the description of immunodeficiencies not yet recognized in veterinary medicine.

## LYMPHOCYTE FUNCTION TEST

Three main functionally different groups of lymphocytes are recognized today, each having more specialized subpopulations: (1) T cells, which have various regulatory and cytotoxic functions in the immune response and hypersensitivity; (2) B cells which may mature to become immunoglobulin and antibody producing cells; and (3) null cells, whose functions are still disputed.

### *Lymphocyte transformation*

The lymphocytes in animals with "normal" immune response can be stimulated by non-specific mitogens or specific antigens to transform (replicate, undergo blastogenesis), whereas lymphocytes of immunodeficient animals will respond in a much lesser degree to the same stimulation. The lymphocyte dysfunction may be caused by lymphocyte defects or serum immunosuppressive factors [62].

The *in vitro* lymphocyte transformation test was developed in the 1960s [63]. Since that time, numerous modifications were made and the test was applied in both research studies and in clinical diagnosis of immunodeficiency. Although there are many variables involved in the test and no absolute values of normal and abnormal transformations are available, the cases of immunodeficiency and immunosuppression may be detected by comparing the results obtained with patient's lymphocytes and those obtained with "normal" animal lymphocytes tested simultaneously. The topic has been reviewed in human [64, 65] and veterinary clinical immunology [62].

In principle, the lymphocytes from the patient's blood (collected in preservative-free heparin or defibrinated with glass beads) are cultured with additions of various mitogens in media containing 10–20% serum from the patient, healthy homologous controls, or fetal calf. After 2–3 days of incubation the blastogenesis is determined by adding  $^3\text{H}$ -thymidine to the cells and culturing them for an additional 18 hr. Then the cells are harvested and washed and the amount of incorporated  $^3\text{H}$ -thymidine is determined in a scintillation counter [62].

The test is quite complex and numerous factors affect the final result. However, when properly performed, a relatively complex and meaningful evaluation of the immune status of the patient's lymphocytes may be obtained. The variables affecting the results are discussed below.

Blood samples should be collected in heparin free of preservatives, or in glass beads containing syringe or dish and defibrinated by shaking. Calcium-free media are necessary for washing cells from heparinized blood. We do not recommend using of whole blood samples for lymphocyte transformation in clinical immunology as has been recommended by some authors [66], because the defects of lymphocytes cannot be distinguished from the suppression due to serum suppressor factors and because the number of lymphocytes may vary considerably in the whole blood samples.

The lymphocytes should be cultured in media containing autologous and healthy homologous and/or fetal calf serum. This allows to determine the presence of lymphocyte immunosuppressive factors in the patient's sera and distinguish this mitogenesis suppression from that caused by the dysfunction of lymphocytes *per se* [62].

The most commonly used mitogens are: Concanavalin A (Con A; in most species a

broad T cell mitogen), phytohemagglutinin (PHA; primarily a T effector cell mitogen), and pokeweed mitogen (PWM; a broad B cell mitogen) [67]. It is recommended to use both optimal (giving peak thymidine incorporation) and suboptimal mitogen doses, because in some defects only the response to suboptimal doses is affected [68].

Use of specific bacterial or viral mitogens has relatively small importance in clinical immunology, because in most cases simpler techniques may detect infection with the given bacteria or viruses. On the other hand, use of non-specific phytomitogens helps to detect the functional status of lymphocytes regardless of the antigen to which they respond.

Optimal timing for maximal thymidine incorporation ranges usually from 2 to 5 days [62, 66, 69, 70], and is affected by numerous factors.

If synthesis of proteins rather than DNA synthesis should be tested, radiolabeled leucine is incorporated into the cells. The major advantages of this technique are its speed, low incidence of contamination, and lack of requirement of serum [71]. However, the latter is also a disadvantage, because this modification does not allow to detect the presence of serum suppressor factors.

### *Lymphocyte enumeration*

The problem of enumeration of various types and subtypes of lymphocytes in domestic animals is far from being resolved to such a degree that these procedures could be used in veterinary clinical immunology testing. Therefore we suggest it to be premature at this point to discuss this topic in the review. This does not mean that the differentiation and enumeration of lymphocytes may not be included in laboratory evaluation of the clinical disorders, however.

### *Interpretation of results*

The potential of lymphocyte transformation test results has not been fully appreciated. The test has to be done in a relatively complex set up with various doses and kinds of mitogens and with additions of various sera to give useful results [62]. For the correct evaluation of results both the background counts per minute (CPM) of unstimulated lymphocytes and the stimulation index or CPM of stimulated lymphocytes must be taken into account. We found the background CPM in clinically healthy dogs varying in considerably smaller range (100–400) than in cattle or horses (200 to over 2000). A mistake would be made by reporting only stimulation indices (i.e. reciprocal of CPM of stimulated cells divided by CPM of unstimulated cells), as well as by reporting only the CPM of stimulated cells. The stimulation indices may be decreased either by decreasing the stimulated cells' CPM and/or by increasing the background CPM. We have found decreased responsiveness of lymphocytes to the phytomitogens in numerous infectious and lymphoreticular diseases caused by either of the following: (1) the presence of serum immunosuppressive factors in the patient serum, (2) the decreased response of lymphocytes *per se* to the mitogens, or (3) the increased mitogenicity of lymphocytes due to unidentified serum factors in absence of phytomitogens [62].

Our preliminary data indicate that at least some infectious diseases are preceded by an increase in the background CPM of unstimulated lymphocytes, which may indicate recruitment of younger cellular forms from the depot of the hematopoietic tissues, and

increased activity and/or numbers of helper or effector cells, all of which are affected by unspecified serum factors in the patient serum. Animals with most infections that we have tested developed in their serum factors suppressing the response of lymphocytes to phytomitogens. The specific target cells for those suppressor factors varied with diseases and with the individual animals. Some viral infections (such as distemper) or chronic bacterial infections (such as brucellosis) affected the response of the lymphocytes *per se*, regardless of the addition of serum to the culture media. Lymphoproliferative tumours (such as bovine enzootic leucosis) caused substantial increase in the proliferation of unstimulated cells and even the serum from infected animals affected blastogenesis of cells from healthy animals.

Our preliminary data indicated that the lymphocyte transformation test results in clinical immunology may detect an incubation period of infectious diseases, a pre-clinical stage of lymphoproliferative diseases, or immunodeficiency of the lymphocytes, which may be either inherited or acquired by infection, but always fatal to the animal. On the other hand, depressed responsiveness of the lymphocytes caused by serum suppressor factors during infections was prognostically favorable and disappeared in relatively short time with the elimination of the infection.

#### DETECTION OF AUTOIMMUNE DISEASES

Autoimmune diseases are characterized by (a) presence of antibodies to "self" antigens in serum; (b) presence of antibodies against modified immunoglobulins (such as rheumatoid factor); (c) deposits of antibodies or incomplete antibodies on blood cells; (d) deposits of antibodies (immunoglobulins) within various tissues.

#### ANTINUCLEAR ANTIBODIES

Antibodies against nuclear proteins or DNA may appear in the blood stream in many autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, and myositis. There are numerous tests used to determine the specificity of these antibodies.

##### *Fluorescent antinuclear antibody test*

The fluorescent antinuclear antibody test [72] is probably the most widely accepted. Though many laboratories use cryostat sections of rodent liver or kidney [73], we had the best results with one of the strains of VERO cell cultures (monkey kidney cells) in testing dog sera (Fig. 10).

The test is a relatively simple indirect immunofluorescent staining procedure. First the patient's serum is placed on tissue cell cultures or cryostat tissue sections. After an incubation at room temperature and washing the unbound protein off, the antibodies from the serum bound to the cellular materials are revealed by staining the culture or section with anti-IgG antibodies conjugated with fluorescein isothiocyanate [72]. The most important factor is the specificity and potency of the anti-IgG serum.

### *Interpretation of the results*

Both the pattern of staining and the titer of the antibodies in patient serum are important. Peripheral staining (Fig. 10a) is typical for active systemic lupus erythematosus (SLE). Diffuse staining (Fig. 10b) appears often with remission of SLE and less often in rheumatoid arthritis. Speckled and nucleolar staining is present in approximately 1/2 of the patients with progressive systemic sclerosis [74]. We suggest titers 40 and above on VERO cell cultures as positive, and titers of 20 as suspect. Lower titers are suggested positive if rodent tissue sections are used [73, 75]. The highest titer of antinuclear antibody detected in our laboratory reached 8000. Dogs with anti-nuclear antibodies at titers 160 and higher were usually severely ill. Peripheral staining with titers 5 on VERO cell cultures was detected in a number of clinically healthy animals in our laboratory.

## RHEUMATOID FACTOR

Rheumatoid factor is an antibody characterized by its ability to react with antigenic determinants present on the Fc portion of the immunoglobulin G (IgG) molecule. It appears in the serum of most but not all patients with rheumatoid arthritis, and in some patients with other autoimmune diseases [76].

### *Rose-Waaler test*

In the 1940s Waaler and Rose and co-workers independently described agglutination of sensitized sheep erythrocytes by sera from patients with rheumatoid arthritis [77, 78]. Modification of their tests is still used under the name Rose-Waaler test in both human and veterinary diagnosis. A test utilizing latex particles as the carrier of immunoglobulin molecules was developed later. These particles were also agglutinated by rheumatoid factor [79]. Unfortunately, this test has not been successfully adapted for testing rheumatoid factor in animals and no commercially prepared latex-IgG particles are available for use in veterinary clinical immunology. From domestic animals, rheumatoid arthritis is most commonly diagnosed in dogs. The topic was recently reviewed [80].

In Rose-Waaler test, sheep erythrocytes are covered with rabbit or dog hemolysin (containing IgG class of antibodies) and reacted with various dilutions of patient serum, which has been pre-absorbed with unsensitized sheep erythrocytes to remove natural antibodies to sheep red blood cells. A control is run simultaneously with unsensitized sheep erythrocytes to detect absence of natural antibodies to the carrier (sheep) cells in the absorbed serum. A differential titer between the agglutination of unsensitized and sensitized sheep erythrocytes is the rheumatoid factor titer [76]. The reading is done macroscopically and under a microscope (Fig. 11). Care has to be taken in the transfer of agglutinated cells, onto the microscopic slide because the agglutinate is relatively weak.

### *Interpretation of results*

Any differential titer exceeding eight is suggested to be positive for rheumatoid factor [76]. Presence of the rheumatoid factor together with clinical symptoms is pathognomic for rheumatoid arthritis or systemic lupus erythematosus with rheumatoid arthritis.



## AUTOIMMUNE HEMOLYTIC DISEASE

Autoimmune hemolytic disease is characterized by the presence of antibodies or C3 (the third component of the complement system) on the patient red blood cells and sometimes even in the patient serum. These antibodies are most often so-called "incomplete" antibodies which do not cause agglutination. They and C3 on the erythrocytes may be detected by adding specific anti-IgG or anti-C3 antiserum to the cell suspension.

### *Direct antiglobulin (Coombs) test*

In the 1940s Coombs and co-workers [81] developed a test for detecting presence of non-agglutinating antibodies to various erythrocyte antigens which may be detected on the patient's red blood cells or even in blood serum in numerous autoimmune hemolytic anemias. Thus a modification of the original antiglobulin (Coombs) test is used in the diagnosis of autoimmune anemias [82].

In principle, red blood cells from blood collected with EDTA (ethylenediamine tetraacetate) are reacted with specific rabbit or goat antiserum against IgG and/or C3 of the species tested for autoantibody. If the cells contain IgG or C3 molecules on their surface, agglutination occurs. A 2% suspension of erythrocytes is usually used, i.e.  $4 \times 10^8$  cells/ml. The rabbit or goat anti serum is diluted in two-fold or four-fold dilutions from 1:2 or 1:4 up. The degree of agglutination may be assessed macroscopically with a magnifying glass or under a microscope (Fig. 11). The critical point is the quality and strength of the antiserum. Care should be exercised in transferring the cells for microscopic observation because the agglutinate is relatively weak.

### *Indirect antiglobulin (Coombs) test*

In this test the presence of antibodies against erythrocytes is detected in the patient serum by using blood group matched donor erythrocytes as the target cells for the antibody [82]. Donor erythrocytes with "incomplete" patient antibody are then treated with the anti-IgG serum as in the direct test to reveal the presence of "incomplete" antibodies on their surface. Because blood group determination or cross-matching is a necessity for this test and because only some patients with autoimmune hemolytic diseases do have detectable circulating antibody, this test is less frequently used in veterinary medicine.

### *Detection of "cold" agglutinins*

A special temperature regime has to be implemented for handling the blood sample to detect so-called "cold" agglutinins which appear more frequently in patients with acquired secondary anemias [82, 83]. The "cold" agglutinins are able to agglutinate the cells at 20 and 4°C but not at 37°C and are "complete" antibody not requiring the use of anti-IgG sera.

### *Interpretation of results*

Because the strength of the reaction depends on both the strength of the antiserum and the concentration of non-agglutinating antibodies on the cells, each laboratory has to

determine the meaning of the various titers of the given antiserum. With commercially prepared antisera used in our laboratory, healthy animals had no detectable agglutination, animals with hemolytic diseases had either undetectable antibodies on their red blood cells (if the disease was not immunologically mediated) or varying titers from four up, but seldom exceeding 100. It must be emphasized that the titer is a reciprocal of the dilution of the anti-IgG or C3 antiserum which only reflects the density of autoantibodies on the red blood cells tested. The so-called "Coombs reagent", which should contain antibodies to both IgG and C3, is commercially prepared for testing dog red blood cells, but may give negative results in some cases when either anti-IgG or anti-C3 sera alone will give agglutination. Thus we recommend using all three types of antisera when autoimmune hemolytic anemia is highly suspected.

In autoimmune hemolytic anemia usually only anti-IgG serum will agglutinate the cells, whereas in other drug-induced hemolytic anemias, usually only anti-C3 antiserum will agglutinate the cells. In anemias associated with systemic lupus erythematosus, both anti-IgG and anti-C3 antisera usually caused agglutination of patient's erythrocytes. On the other hand, in anemias associated with presence of "cold" agglutinins anti-C3, but not anti-IgG, will give agglutination of patient's red blood cells [82].

#### AUTOIMMUNE ORGAN OR TISSUE DISORDERS

Many immunologically mediated diseases are associated with either production of antibodies against the own patient's tissue antigens or deposits of immune complexes of antibodies and antigens in the tissues. Renal [84] and skin [85] biopsies are the most commonly tested tissues by direct immunofluorescence. Donor healthy tissues may be used to detect presence of circulating antibodies by indirect immunofluorescence in a number of autoimmune diseases [86].

##### *Direct immunofluorescence technique*

In the direct staining procedure, the cryostat (frozen) tissue sections from the patients are stained, after thawing at room temperature, with specific anti-IgG, anti-IgM, anti-IgA, or anti-C3 antibodies conjugated with fluorescein isothiocyanate (FITC) stain. After rinsing and mounting, the sections are observed under a fluorescent microscope and the staining intensity and pattern are recorded [84, 85] (Fig. 12a).

##### *Indirect immunofluorescence technique*

In the indirect staining procedures, the cryostat tissue section from healthy donors are first treated with patient's serum and after rinsing stained with specific FITC anti-IgG. The anti-IgG antibody will stain only those locations where the patient's serum sample deposited its specific antibody against the tissue constituents [86] (Fig. 12b).

Adequate tissue sections, optimal fluorescent antibody reagents, adequate fluorescent microscopic equipment, adequate controls, and a good deal of experience in interpreting the results is necessary for satisfactory immunofluorescence study.

### Interpretation of results

The detailed discussion on the interpretation of results is beyond the scope of this overview. In direct staining, one has to take into account the clinical stage and the development of the disease, the area of sample collection and the results of the control healthy animal samples treated simultaneously.

In autoimmune glomerulonephritis due to autoantibodies to glomerular basement membrane, linear deposits of IgG and C3 (in approximately 1/3 of the patients) are the most common features [87]. In glomerulonephritis caused by immune complex diseases, the deposits of IgG and C3 are granular and usually on the epithelial side of the membrane [88]. In systemic lupus erythematosus both linear and granular deposits may be detected because both direct antiglomerular antibodies or immune complexes may develop [84].

In autoimmune dermatologic diseases, direct immunoflorescent staining of the skin sections and indirect staining using the patient's serum may differentiate various types of skin disorders [89–91]. Direct staining of intercellular cement substance and presence of circulating antibodies to these substances (detectable by indirect immunofluorescence) is typical for pemphigus vulgaris. The basement membrane at the dermal-epidermal junction stains in patients with bullous pemphigoid disease, in which also circulating antibodies to the basement membrane are often detectable (Fig. 12). Dermatitis herpetiformis is characterized by more intense staining of the basement membrane with FITC anti-IgA than FITC anti-IgG. The skin basement membranes also stained with FITC anti-IgG in systemic lupus erythematosus, but the staining is usually granular as compared to a linear staining in bullous pemphigoid disease. Neither in dermatitis herpetiformis nor in systemic

Table 3. Presence of autoantibodies in various immunologically-mediated diseases

| Antibodies against tissue                          | Disease                                                                                                                        | Reference     |
|----------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------|---------------|
| Thyroid (thyroglobulin)                            | Thyroiditis (40–70% patients)<br>Thyrotoxicosis (less than 40%)<br>Non-toxic goiter (small percentage)                         | 92, 93, 94    |
| Microsomal antigen of thyroid epithelial cells     | Chronic thyroiditis (up to 90%)<br>Primary hypothyroidism (64%)<br>Thyrotoxicosis (50%)<br>Simple goiter (10%)                 | 95            |
| Adrenal antigens                                   | Idiopathic Addison's disease (38–60%)<br>Tuberculous Addison's disease (7–18%)<br>Normal subjects (1%)                         | 96            |
| Parathyroid (cytoplasmic antigen)                  | Idiopathic hypoparathyroidism (38%)<br>Idiopathic Addison's disease (26%)<br>Chronic thyroiditis (12%)<br>Normal subjects (6%) | 86, 97        |
| Mitochondrial antigens (thyroid or kidney tissues) | Primary biliary cirrhosis (87–98%)<br>Active chronic hepatitis (25–28%)<br>Cryptogenic cirrhosis (25–30%)                      | 98            |
| Smooth muscle                                      | Active chronic hepatitis (40–70%)<br>Primary biliary cirrhosis (50%)<br>Cryptogenic cirrhosis (28%)<br>Normal subjects (2%)    | 99, 100       |
| Islet cells of pancreas                            | Insulin-dependent diabetes mellitus (60–85%)<br>Normal subject (0.9–1.7%)                                                      | 101, 102, 103 |

lupus erythematosus circulating antibodies could be detected by indirect immunofluorescence [89]. Both direct and indirect immunofluorescence of dog skin was used in diagnosis of this disease in veterinary medicine [90, 91].

Autoantibodies to various other body tissues may be detected in diseases given in Table 3.

### DETECTION OF ALLERGIC DISEASES

Various sensitive tests quantitating IgE in man have been developed to detect allergic patients with elevated IgG. Unfortunately, attempts to isolate larger quantities of purified animal IgE molecules and to prepare the necessary specific antisera were unsuccessful so far. Thus there are no commercially prepared tests available to detect IgE levels in animals, as there are none practically used to detect type IV hypersensitivities in animal patients.

Fortunately, both hypersensitivities type I and type IV may be tested relatively efficiently *in vivo* by intradermal administration of various commercially prepared allergens.

Because hypersensitivity reactions type II are the consequences of autoimmune diseases and type III are consequences of some of the immune reactions, there is no special laboratory testing practical for those types of hypersensitivities.

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