ANALYSIS OF NORMAL CAT SERUM BY IMMUNOELECTROPHORESIS

Shin OKOSHI, Isamu TOMODA and Susumu MAKIMURA

*Department of Veterinary Internal Medicine and Parasitology,
Faculty of Agriculture, University of Tokyo, Tokyo*

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Studies have been made on the analysis of cat serum proteins under normal conditions by using the moving boundary and the zone electrophoretic methods^{1,2,5,7,8,12,14,16,18}. GROULADE et al.⁸⁾ reported age variations in the serum proteins, glycoproteins, and lipoproteins. Most of those studies, however, are limited in methods employed. No electrophoretic studies have been performed on the pathological serum proteins of cats. At the authors' laboratory, attempts have been made to apply the zone electrophoretic methods and immunoelectrophoretic techniques to small animal practice. The normal dog serum proteins have already been analyzed by immunoelectrophoresis, as mentioned in the previous paper¹⁵⁾. In the present investigation, the normal cat serum proteins were analyzed by the zone electrophoretic and immunoelectrophoretic methods. In addition, comparison was made between the normal human and cat serum proteins, and the cat, dog and human serum proteins were tested for cross-reaction.

MATERIALS AND METHODS

Fifty healthy cats, including newborns and adults, were used (Table 1). None of them harbored eggs of any intestinal parasite in the feces. The kittens newborn at birth and one to forty days of age had been born from healthy mother cats, showing steady and satisfactory growth. In addition, four fetuses taken from a healthy mother cat by cesarotomy at about 50 days of fetal life were used as materials.

Blood samples were collected from the adult and young cats usually through the cephalic vein, the recurrent tarsal vein, or the jugular vein, and from the fetuses, newborns, and sucklings by cardiac puncture.

Normal human and dog blood samples were collected from healthy individuals.

Table 1. Cats Used as Materials

Group	Age	No. of cats	Total protein* (g/dl)
Fetus	About 50 days	4	3.1 $\pm$ 0.1
Newborn	Precolostral, 12 hours	3	3.3 $\pm$ 0.1
1	2 to 3 days	10	3.6 $\pm$ 0.3
2	5 to 7 days	5	3.9 $\pm$ 0.2
3	2 to 3 weeks	6	4.3 $\pm$ 0.2
4	1 to 2 months	5	4.7 $\pm$ 0.1
5	3 to 5 months	6	5.3 $\pm$ 0.3
6	6 to 10 months	5	6.4 $\pm$ 0.2
7	Adult, over 1 year	10	6.8 $\pm$ 0.3

* Mean value and standard deviation.

The blood samples were allowed to stand and clot at room temperature for about 30 minutes and centrifuged at 3,000 rpm for minutes. The resultant sera were stored at -20°C when not in use. Zone electrophoresis was performed on all the serum samples either immediately after separation from the clot or after storage in frozen state for within 5 days. Immunoelectrophoresis was carried out on serum samples which had not been frozen. Blood samples were discarded when hemolysis had occurred to them.

Rabbits were used to prepare antiserum against cat whole serum. They were inoculated subcutaneously with eight doses of 1.5 ml each of normal cat whole serum with an equal amount of Freund's complete adjuvant (Iatron Laboratories, Tokyo) over a period of 2 months. The resulting antiserum was collected on the 10th and 15th day after the final inoculation, and stored in thimerosal (1:10,000) at -20°C .

The anti-dog serum employed was the same as used previously¹⁵. The rabbit antisera against human whole serum, albumin, transferrin, and 7S-globulin used were all commercially products (Behringwerke, AG., Marburg-Lahn, Germany).

Absorption of antiserum was made by adding one volume of an antigen to four volumes of antiserum. The mixture was incubated at 37°C for 3 hours and centrifuged. The resulting supernatant was used as absorbed antiserum.

Immunoelectrophoresis and cellulose-acetate, paper, and agar gel electrophoresis were carried out by the same methods and techniques as described in the previous paper¹⁵.

For the staining of protein, ponceau 3 R was used in cellulose acetate electrophoresis, and Amidoshwartz 10 B in paper and agar gel electrophoresis. Immunoelectrophoretic slides were routinely stained for protein with Amidoshwartz 10 B or nigrosin. A few of them were stained for lipoproteins with Sudan black B and for glycoproteins with Schiff's reagent. To demonstrate oxidase and peroxidase activity, paraphenylenediamine and *o*-dianisidine were used, respectively.

The stained cellulose acetate strips were scanned with a recording densitometer, model Densicord 542 (Photovolt Ltd., U.S.A.). The percentages of individual fractions were calculated by counting the number of teeth on the integrating lines of Densicord patterns. Protein concentrations were determined by using a hand protein refractometer, model PRP (Hitachi Ltd.).

RESULTS

Paper electrophoresis using the conventional veronal buffer pH 8.6 and 0.1 ionic strength divided the normal adult cat serum protein into five distinct components. It was possible to designate the five components of the serum protein as albumin, α_1 -, α_2 -, β -, and γ -globulin respectively, according to mobility and by comparison with normal human serum protein patterns. The α_2 -globulin fraction was subdivided into two components, a rapid and a slow, in all the cases, except a few. The results of cellulose acetate electrophoresis showed that the α_2 -globulin fraction could be subdivided into two components in all sera. Accordingly, it was indicated by this method that the normal adult cat serum proteins consisted of six distinct components. On the other hand, the results of agar gel electrophoresis showed that the cat serum proteins could usually be separated into the same six components as those demonstrated by cellulose acetate electrophoresis, and that they often had some subdivided components of the α -globulin (Fig. 1). Comparison among the sera indicated that cat serum albumin migrated most rapidly and human serum albumin most slowly. There was a difference in the position of each globulin fraction among cat, dog, and human sera (Fig. 2).

The immunoelectrophoretic pattern of normal adult cat serum developed by anti-normal cat whole serum usually revealed the presence of at least 17 and at most 23 precipitin

lines, including very faint ones. The protein components of cat serum were designated by the nomenclature system proposed in a previous paper¹⁵. Chart 1 is a diagrammatic presentation of all the precipitin lines observed by immunoelectrophoresis. Six groups, consisting of albumin and α_1 -, α_2 -, β_1 -, β_2 -, and γ -globulin, were observed. Twenty-three antigenic components, one in albumin, six in α_1 -globulin, nine in α_2 -globulin, two in β_1 -globulin, four in β_2 -globulin, and one in γ -globulin, were demonstrated in normal adult cat serum.

Cat serum stained with Sudan black B showed three lipoprotein lines corresponding to albumin, α_1 -II, and α_2 -VII. The lipoprotein stain of the albumin and α_1 -II lines was relatively strong in comparison with the α_2 -VII line. On the other hand, 14 lines of glycoprotein were exhibited by staining with Schiff's reagent. Staining of normal adult cat serum with paraphenylenediamine did not show any definite uptake of brown pigment by any of the identified precipitin lines. By the benzidine test of normal cat serum mixed with hemoglobin, weak peroxidase activity observed in the bands corresponding to the α_2 -I and β_1 -I. Simple agar gel electrophoresis of the same serum showed the presence of strong peroxidase activity in the region of the α_3 - and β_1 -globulin fractions.

Immunoelectrophoresis of normal human serum developed against anti-cat serum indicated the presence of three lines corresponding to the albumin, α_2 -I, and β_1 -I components, respectively. On the other hand, immunoelectrophoresis of normal cat serum developed against anti-human serum showed the presence of three precipitin lines. By their mobility and shape, these three lines were assumed to correspond to the albumin, α_2 -I, and probably β_2 -I-lines, respectively. The cross-reaction between normal cat and human sera is outlined in Chart 2 C and H. Normal cat serum cross-reacted with anti-dog serum, producing five arcs (Chart 2 B). It was reasonable to presume that these five arcs corresponded to the albumin, α_1 -IV, α_2 -I, α_2 -VI, and β_1 -I lines. Immunoelectrophoresis of normal dog serum indicated the presence of four precipitin lines when tested against anti-cat serum (Chart 2 E). These four lines seemed to correspond to the albumin, α_1 -IV, α_2 -I, and β_1 -I lines, respectively.

There were differences in the electrophoretic mobility of the protein components among normal cat, dog, and human sera, as mentioned above. There were close similarities, however, in the number, shape, and characters of the protein components among these sera. Therefore, the antigenic components were identified and designated by comparing the immunoelectrophoretic pattern among normal cat, dog, and human sera with regard to the special staining characteristic, mobility, and position of each line. The number of precipitin lines usually found in normal adult cat serum and the bands in normal human serum seemed to correspond to these lines are listed in Table 2.

Changes in the serum proteins were scrutinized in various stages of life ranging from the fetus to the adult. In the sera of fetal and precolostral newborn kittens, no γ -globulin

Chart 1. Diagrammatic Representation of Immunoelectrophoretic Pattern of Normal Adult Cat Serum Developed against Rabbit Antiserum to Cat Whole Serum

The figure in parentheses gives the number of precipitin lines found in each mobility range.

Chart 2. Diagrammatic Representation of Results of Immunoelectrophoresis for Cross Reaction among Normal Cat, Dog, and Human Sera

Remarks. A—C: normal cat serum vs. (A) anti-normal cat serum, (B) anti-normal dog serum, and (C) anti-normal human serum.
 D—F: normal dog serum vs. (D) anti-normal dog serum, (E) anti-normal cat serum, and (F) anti-normal human serum.
 G—I: normal human serum vs. (G) anti-normal human serum, (H) anti-normal cat serum, and (I) anti-normal dog serum.

fraction was observed by electrophoresis. In their immunoelectrophoretic patterns, the sera of fetal and precolostral newborn kittens failed to show such γ -globulin line as corresponding to γ G-globulin. In all sera collected 12 or 24 hours after birth, the γ -globulin fraction and arc appeared in a significant manner. The γ -globulin concentration showed no significant change in the sera of colostrum-fed newborn kittens almost up to 10 days of age, but exhibited a tendency to begin to decrease gradually at about the 2nd week of age. The γ -globulin fraction showed an excessive decrease in concentration at one to two months of age, but it began to rise at about three months of age (Table 3). In the sera of adult cats, especially older ones, a very long line indicating the γ -globulin components and running into the α -globulin area was occasionally observed.

In addition, the β_2 -I and β_2 -III arcs, corresponding to γ A- and γ M-globulin, respectively, were absent in fetal and precolostral newborn kitten sera. In sera from some suckling more than two weeks of age, the lines representing these arcs were observed when examined under suitable conditions, although they were very faint. Moreover, in the β_2 - and γ -

Table 2. Precipitin Lines in Immunoelectrophoretic Pattern of Normal Adult Cat Serum, Staining Characteristics, and Comparison with Lines in That of Human Serum

Precipitin line in cat serum	Characteristic staining	Probably corresponding line in human serum
Albumin	Glycoprotein Lipoprotein	Albumin (Lipoalbumin)
α_1 -I	Glycoprotein	α_1 -glycoprotein
α_1 -II	Lipoprotein	α_1 -lipoprotein
α_1 -III	Glycoprotein	Orsomucoid
α_1 -IV	Glycoprotein	
α_1 -V		
α_1 -VI		
α_2 -I	Glycoprotein Peroxidase activity	Haptoglobin
α_2 -II		
α_2 -III	Glycoprotein	Ceruloplasmin
α_2 -IV	Glycoprotein	α_2 -macroglobulin
α_2 -V		
α_2 -VI		
α_2 -VII	Lipoprotein	α_2 -lipoprotein
α_2 -VIII		
α_2 -IX		
β_1 -I	Glycoprotein	Transferrin
β_1 -II	Glycoprotein Peroxidase activity	Hemopexin
β_2 -I	Glycoprotein	γ A
β_2 -II	Glycoprotein	
β_2 -III	Glycoprotein	γ M
β_2 -IV	Glycoprotein	
γ	Glycoprotein	γ G

Table 3. Average Concentrations of Serum Protein Components in Age Groups of Normal Cats as Determined by Cellulose Acetate Electrophoresis

Group*	Protein components (%)**						A/G ratio**
	Albumin	Globulin					
		α_1	α_2	α_3	β	γ	
1	38.7±3.1	9.0±2.8	11.1±1.1	11.5±2.8	13.5±1.7	16.2±2.4	0.63±0.09
2	40.6±1.8	7.3±0.8	11.8±1.7	10.6±0.9	13.0±0.7	16.7±2.4	0.68±0.05
3	45.1±2.8	5.8±0.5	10.7±0.9	10.7±1.0	13.6±1.8	14.1±2.5	0.82±0.10
4	47.9±4.1	4.1±1.8	11.6±1.0	14.0±1.2	15.6±1.1	6.8±2.6	0.92±0.13
5	44.5±2.3	4.3±3.0	8.6±0.7	11.6±2.3	12.8±2.7	18.1±2.4	0.80±0.08
6	44.2±2.5	2.2±0.9	7.6±1.2	14.1±2.5	13.4±2.2	18.5±2.3	0.70±0.08
7	37.7±3.5	4.4±1.8	8.0±1.6	12.6±3.0	12.8±2.6	24.5±3.1	0.60±0.10

* See Table 1.

** Mean value and standard deviation.

globulin areas of adult cat serum, additional two or three faint and obscure arcs were visualized occasionally.

DISCUSSION

Attempts have been made by a multitude of investigators to use the determination of serum protein levels as an aid for the diagnosis of a disease. A physical, chemical, or immunological method has been tried on available pathological sera, in the hope of

developing an effective diagnostic test. To identify the components of pathological serum, it is necessary to determine the pattern produced by normal serum. Electrophoretic analysis of normal cat serum has been performed by numerous investigators, who identified the following numbers of components: 5 to 7 by moving boundary, up to 7 by paper, and up to 16 by starch gel electrophoresis⁷⁾. In the present studies, normal cat serum was separated into at least five major fractions by paper electrophoresis, and six components by cellulose acetate electrophoresis. Agar gel electrophoretic analysis effected good resolution of the same six basic components as observed by the cellulose acetate method and often produced separation of additional components. On the other hand, analyses on normal cat serum proteins from healthy individuals indicated that the five to seven conventional components identified by zone electrophoresis as mentioned above could be separated into 23 antigenic constituents by immunoelectrophoresis developed with the authors' antiserum to normal whole serum.

There are many so-called normal values for the various serum proteins of cat. A wide variation, however, is observed in the acceptable normal values. It is generally agreed that there will be difference in quantitative values of the components of serum protein and that this difference depends greatly on many factors, such as strain, care, age, sex, and diet¹⁸⁾. In addition, the values vary appreciably with different methods or investigators even in the same sample. Accordingly, the determination of normal values should be made from an accumulation of data from a significant number of healthy subjects which are all maintained under similar conditions. It is important that particular attention should be paid to the method of analysis and that the same technique should be used throughout an experiment. In this investigation, mean percentage values of the serum proteins were determined in normal adult cats one to five years of age by cellulose acetate electrophoresis. They were as follows: albumin, 37.7 ± 3.5 ; α_1 -globulin, 4.4 ± 1.8 ; α_2 -globulin, 8.0 ± 1.6 ; α_3 -globulin, 12.6 ± 3.0 ; β -globulin, 12.8 ± 2.6 ; and γ -globulin, 24.5 ± 3.1 . These values were in good agreement with those reported by GROULADE et al.⁸⁾ who had used paper electrophoresis.

The proteins of serum are affected by age. Various studies have been made on the significance of age and the concentrations of the serum proteins⁶⁾. Generally, albumin decreases in concentration and the globulin fractions increase with the advance in age. Especially, it is of interest to note that the γ -globulin fraction varies with age and with species. In human, monkey, rabbit, and guinea pig sera, the γ -globulins are transmitted prenatally and found in the serum of the newborn in a normal amount. In cows, goats, sheep, horses, and pigs, the serum of the newborn contains only traces of γ -globulin, if any. On the other hand, it is agreed that the transmission of antibody and γ -globulin from mother to young may occur both before and after birth in rats, mice, dogs, and probably cats. In dogs, however, γ -globulin was contained neither in fetal nor in precolostral newborn puppy serum, but a considerably large amount of γ -globulin appeared in suckling puppy serum¹⁵⁾. In the present study on the changes of the serum proteins in cats with age, a changes similar to the above mentioned was also observed. The γ -globulin fraction of the serum was found neither in the fetus nor in the precolostral newborn of cats, but in kittens 12 to 24 hours of age in a significant amount. HARDING et al.⁹⁾ mentioned that the kittens born from mother cats immunized against *Salmonella montevideo* showed titers of about 1:200 in the precolostral stage, and 1:1000 after nursing, and remained at this level of titer during the first week of life. Therefore, as mentioned in the previous paper¹⁵⁾, it seemed reasonable to assume that very little, if any, γ -globulins might have been transmitted from the mother to the fetus by transplacental transfer, but that a significant amount of the γ -globulins appeared in the fetus within a few hours after birth as a result of absorption of the γ -globulins contained in the colostrum.

MOORE¹²⁾ reported that γ -globulin was at a significantly low level in a 4-day-old kitten. OTTEN et al.¹⁶⁾ mentioned that the duration of hypogammaglobulinemia seemed to extend from the day of birth to 75 days of age. On the other hand, GROULADE et al.⁸⁾ asserted that γ -globulin began to show a decrease on the first day of life and seemed to reach its minimum value some time between 15 and 45 days of age. These data were virtually identical with those mentioned above, but there were no significant changes in γ -globulin concentration in colostrum-fed newborn kittens up to 1 week of age. In the above-mentioned and the present studies, the determination of serum protein changes with age was not carried out on the same samples. Naturally, many difference appeared in the serum protein levels of healthy cats even of the same age. As compared electrophoretically with sera of colostrum-fed kittens one to two days of age, sera of most 7-day-old kittens showed no changes in any individual fraction, except a some-what lower concentration of the γ -globulin fractions. No significant variation in γ -globulin level, however, was seen among colostrum-fed kittens 1, 2, 5, and 7 days old born from the same mother cats. Consequently, it was believed that the changes of serum protein observed in the present investigation showed the rough tendency of the γ -globulin fractions to develop from the fetus to the adult stage in cats.

It is generally agreed that there are considerable differences in electrophoretic mobility of an individual fraction among sera of various animals⁴⁾. DEUTSCH and GOODLOE⁵⁾ and KOBAYASHI et al.¹¹⁾ found that serum albumin migrated proportionally in the cat further than in other animal species. GROULADE et al.⁸⁾ concluded that cat serum and plasma showed a greater mobility of the protein fractions than human ones. In this investigation, cat whole serum also showed such individual characteristics as the faster mobility of albumin and larger amounts of α - and γ -globulin fractions than dog and human sera. However, the number of precipitin lines and their characters revealed by immunoelectrophoresis were very similar between cat and dog sera.

Cross-reaction was demonstrated by immunoelectrophoresis between cat and human sera, cat and dog sera, and dog and human sera. Especially, the most numerous and distinct arcs were observed by the immunoelectrophoretic methods when normal cat serum had been subjected to cross-reaction with anti-dog serum, and normal dog serum with anti-cat serum. PORTER and DIXON¹⁷⁾ reported that 8 precipitin bands appeared in the reaction between normal mink serum and anti-human serum, while 4 precipitin arcs did in the reaction between anti-normal mink serum and human serum. It was also demonstrated that normal sheep and pig sera revealed cross-reaction with antisera of many kind. KAMINSKI¹⁰⁾ found that normal sheep serum exhibited crossreaction with anti-human sera prepared from a number of hosts, producing one to eight arcs of precipitation. CHORDI and KAGAN⁸⁾ suggested that the cross-reaction between human haptoglobin, albumin, and γ -globulin with normal sheep serum might be worth while making comment because of the possibility of inducing a nonspecific reaction in serodiagnostic tests of hydatidosis, in which sheep or pig hydatid fluid is commonly used as antigen. Accordingly, it was felt that the cross-reactions observed in this investigation were of importance in relation to the serologic diagnosis. On the other hand, it may be proved from the results of immunoelectrophoresis of normal cat serum developed against antihuman serum or anti-dog serum that cat serum will differ qualitatively, and at least immunochemically, in most of the protein components from dog and human sera.

Electrophoretic methods have made it possible to detect and determine serum protein changes during the fetal and postnatal developement in several species. Recent application of the immunoelectrophoretic technique has enabled to expand the knowledge of qualitative changes in serum protein pattern during the stage of fetal development. The fetuses used in this investigation were about fifty days of fetal life. In consequence, immunoelectro-

phoresis of their serum proteins gave entirely the same patterns as observed in the precolostral newborn kitten serum proteins. It was demonstrated by immunoelectrophoresis of normal adult serum developed against anti-cat serum absorbed with fetal or precolostral newborn kitten serum that in both sera, there were many fractions which corresponded immunologically to the adult serum fractions. However, it was thought advisable to investigate the serum protein fractions of cat fetuses at various stages of development. Moreover, the antisera used in the present study had been directed toward adult serum. Therefore, it was reasonable to presume that no fetus-specific proteins had been detected. Accordingly, it was believed that the use of purified antigens prepared from fetal material was required to clarify any true changes of the serum protein components with the advance in fetal development.

SUMMARY

Normal adult cat serum was analyzed by electrophoretic and immunoelectrophoretic techniques. Conventional five protein fractions were identified by paper electrophoresis, six by cellulose acetate electrophoresis, at least nine, including some weak bands, by agar gel electrophoresis, and 23 by immunoelectrophoresis. Some specific staining characteristics of the precipitin lines were investigated.

A standard system of nomenclature was proposed to identify the antigenic components revealed by immunoelectrophoresis and compare them with the bands observed in human serum by electrophoresis. The number of antigenic components and their characters were very similar among human, dog, and cat sera, especially between dog and cat sera, although there were differences in electrophoretic mobility among the three sera. On the other hand, there was immunologic cross reactivity between human and cat sera, or dog and cat sera.

In addition, the sera of cats, including fetuses, the newborn, and adults, were analyzed. It was of interest to note that variation in the γ -globulin fraction with the advance in age was somewhat similar between cat serum and the dog serum examined in the previous study.

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猫血清蛋白に関する免疫電気泳動的研究

大越 伸・友田 勇・牧村 進

東京大学農学部家畜内科学及寄生虫病学教室

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健康成猫血清蛋白は、濾紙電気泳動法、セルローズ・アセテート電気泳動法、および寒天電気泳動法によって、5ないし9分画に分離された。猫血清蛋白各分画の電気泳動相対移動度は、人および犬血清蛋白の移動度にくらべて、若干の相違があり、特に、猫血清 albumin は、最も早く移動することを認めた。

免疫電気泳動法による分析では、著者らの家兎抗血清を使用した場合、微細な、やや不鮮明なものを含めて、最大 23 本の沈降線を検出できた。これらの沈降線の命名は、人血清蛋白電気泳動像と対比

しながら、CHORDI らの方法に従った。さらに、Amidoschwartz 10 B による蛋白染色に加えて、Schiff 試薬, Sudan black B, *o*-dianisidine, para-phenylenediamine に対する特異染色性について検討した。また、猫血清と人および犬血清との間の交叉反応についても観察した。

一方、猫血清蛋白の年令的変動を、セルローズ・アセテート電気泳動法と免疫電気泳動とによって観察し、特に γ -globulin 分屑の動態について検討を行なった。

EXPLANATION OF PLATE

PLATE I

Fig. 1. Electrophoresis of normal cat serum.

Upper: immunoelectrophoretic pattern. Lower: agar gel electrophoretic pattern.

Fig. 2. Cellulose-acetate electrophoretic patterns of normal cat, dog, and human sera showing difference in electrophoretic mobility of the serum protein fractions.

H: human serum, C: cat serum, D: dog serum, H+C: cat serum with an equal amount of human serum, C+D: cat serum with an equal amount of dog serum, and H+D: dog serum with an equal amount of human serum.

Fig. 1

Fig. 2