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Neutralizing Antibody to Viruses of Poliomyelitis in Sera of Domestic Animals. (22109)

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The explanation for the origin of seasonal outbreaks of poliomyelitis in the temperate zone is unknown, although it is now evident that man to man transmission is responsible for maintenance of an epidemic. Viral isolation studies have, as yet, failed to implicate animal-reservoir-insect-vector pattern which is so characteristic of many other neurotropic viruses. However, most isolation studies were done prior to the development of tissue culture technics. and intensive surveys were limited. Immunological studies have been carried out by Hammon(1) and others(2,3) in which "neutralizing factors" or "protective substances" have been found in sera of various animals. The status of these "protective substances" is uncertain for they were demonstrable only in low dilution and against one strain (Lansing, type 2), the only strain readily available in most laboratories. Whether one is here dealing with a non-specific neutralizing substance or specific antibody is a critical point, for identification of these substances as specific antibodies to viruses of poliomyelitis would point strongly to the existence of either (1) an extra-human reservoir of viruses of poliomyelitis or (2) a serologically related group of viruses. In the present study animal sera were tested for presence of neutralizing antibody to all 3 types of poliomyelitis virus using the HeLa cell. The protective substance existing in certain cases was identified as a true antibody. To determine the origin of this antibody, extensive though unsuccessful attempts were made to isolate the agents responsible for production of this antibody.

Methods. The HeLa Cell. Techniques employed in the management of the HeLa cell have been adequately described(4), and were followed in the present study. The HeLa cells were routinely grown in rubber stoppered test tubes in a growth medium consisting of 40% human adult serum, 2.5% chick embryo extract and 57.5% Hanks' balanced salt solution. Before use in neutralization test, the cells were washed 3 times with balanced salt solution and 0.9 ml maintenance solution was added to each test tube. The maintenance medium was composed of 90% maintenance solution as described by Syverton, Scherer and Ellwood(4) and 10% chicken serum. *Strains of Poliovirus.* The Brunhilde (type 1), YSK (type 2) and Leon (type 3) strains were employed as representatives of the 3 known antigenic types of poliovirus. These viruses were used in a 100 tissue culture dose concentration against each animal sera tested. *Source and Preparation of Serum Samples.* Table I illustrates the source of sera and geographical origin of animals from which sera were obtained. Sam-

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TABLE I. Number and Source of Animal Sera.

Animal	No. of sera	Source	Geographical origin
Cows	20	Slaughter house	Pa.
Steer	22	*Idem*	Midwest
Calves	18	"	Va.
Horses	28	Vet. School, Univ. Pa.	Pa. & N.Y.
Hogs	19	Slaughter house	Midwest
Chickens	20	Poultry market	Pa.
Lambs	19	Slaughter house	Va.
Dogs	26	Temple Med. School	Pa.
Cats	5	*Idem*	Unknown

ples of blood were collected in sterile tubes, allowed to clot, and refrigerated overnight. Serum was drawn off, inactivated at 56°C for one hour, and stored in a dry-ice box for approximately 6 months before testing. *Neutralization Test.* Serum dilutions were placed in sterile test tubes in 0.4 ml amounts to which was added 0.4 ml virus, of either types 1, 2 or 3, containing 100 tissue culture doses. The serum-virus mixtures remained at room temperature for one hour. Neutralization of the virus was determined by inoculating 0.1 ml of the mixture into 3 HeLa tubes. Tissue, animal serum toxicity and virus controls were routinely run. After 96 hours at 36°C the tubes were read for cytopathogenic effects. Highest dilution of serum completely inhibiting action of virus was the neutralizing titer of the serum. To determine if the neutralizing action was associated with the globulin fraction of the sera, globulins were precipitated out with a saturated solution of ammonium sulfate. The precipitated globulin was then diluted with distilled water to a final volume equal to that of original volume of sera. This was dialyzed and tested to determine if it had retained its protective activity against viruses of poliomyelitis. *Sources and Preparation of Tissue Specimens for Viral Isolation Studies.* In an attempt to establish the presence of a virus that might explain the origin of antibodies to viruses of poliomyelitis, samples of spinal cord, spleen, lymph node and feces were collected from cows, steers and calves. Specimens were collected as follows: approximately 1 cm^2 tissue, or 10 ml of feces were collected, 10 specimens from 10 animals pooled, and a 50% suspension prepared by grinding with mortar and pestle in tryptose broth containing 300 units/ml penicillin and 300 μg/ml dihydrostreptomycin. The suspension was then centrifuged at 3500-4000 rpm for 30 minutes, and the supernatant fluid tested for viral activity. Some tissue specimens proved to be toxic to HeLa cells, therefore the supernatant fluid of centrifuged suspensions was further diluted 1-5 in Hanks' balanced salt solution to reduce toxicity of test materials. One-tenth ml of each specimen was inoculated into 3 HeLa cultures in test tubes containing 0.9 ml maintenance solution. These tubes were incubated at 36°C and observed for cellular changes. After 5 days the fluid medium from each set of 3 cultures was pooled and 0.1 ml of this was inoculated into each of 3 HeLa cultures. This second passage also served further to dilute out toxic factors. After 4 days incubation at 36°C HeLa cultures were again observed for cytopathogenic changes. In addition, tissues were stained and observed for inclusion bodies to detect any agent which might have multiplied without producing a gross cytopathogenic effect. After removal of the media, HeLa cultures were stained directly in test tubes and on coverslips from Porter flask cultures with May-Grunwald-Giemsa stain.

Results. The data presented in Table II indicate type and number of animal sera having neutralizing activity against poliovirus. At a serum dilution of 1:2 some in-

TABLE II. Neutralizing Activity of Animal Sera against Polioviruses Types 1, 2 or 3.

Animal	No. of sera tested	No. of animals with neutralizing activity against polioviruses* Neutralization titer 1:2	1:10 or greater
Bovine			
Cow	20	5	14
Steer	22	3	6
Calf	18	5	0
Lamb	19	5	0
Chicken	20	6	2
Hog	19	3	6
Horse	28	6	9
Dog	30	5	0
Cat	5	0	0

* An animal serum was listed as a single positive if it neutralized types 1, 2 or 3 polioviruses or any combination of the 3 types.

TABLE III. Type Distribution of Neutralizing Activity against Poliomyelitis Viruses, Types 1, 2, and 3.

Animal	No. of sera tested	No. of positive reactions* Type 1	Type 2	Type 3	Total No. of positive reactions†
Bovine					
Cow	20	8	12	4	24
Steer	22	1	5	0	6
Calf	18	0	0	0	0
Horse	28	5	6	0	11
Hog	19	2	3	1	6
Chicken	20	1	1	0	2

* Only those sera exhibiting a neutralization titer of 1:10 or greater were included among the positive reactions.

† A single positive serum specimen may have contributed 1, 2 or 3 positive reactions depending on its ability to neutralize 1, 2 or 3 types of poliomyelitis virus.

dividuals of each species tested showed neutralizing activity, with exception of the cat. In many cases this neutralizing activity was lost upon further dilution. Since the neutralizing activity of undiluted or only slightly diluted serum may have been the expression of a non-specific inhibitory substance, only those species whose sera showed at least occasional activity at dilutions of 1:10 or greater were considered as possible poliomyelitis antibody producers. It will be seen in Table II that cows, steers, horses, hogs, and to a questionable extent, chickens possessed a capacity to neutralize one or more of the 3 types of poliomyelitis virus in serum dilutions of 1:10 or greater. On the other hand, sera of calves, lambs, dogs and cats were consistently negative at dilutions of 1:10 or higher, hence were not considered to be related to specific antibody. Type distribution of positive sera in the cow and steer is indicated in Table III. It will be seen that neutralizing activity against type 2 poliovirus was most frequently encountered followed by type 1, and least frequently against type 3.

Probably the strongest evidence indicating that these neutralizing substances are antibodies is the pattern of neutralization observed with cow and steer sera as indicated in Tables IV and V. It can be seen that titers vary from 1:10 to 1:250 (the highest dilution tested) and that the pattern of neutralization tends to differ with each animal. Some animals have antibody to type 1, some to type 2, some to type 3, and still others have various combinations of these antibodies. This pattern, by definition, rules out the possibility of neutralizing action being due to a non-specific inhibitory substance. As further evidence that these are antibodies resulting from a specific antigenic stimulus and not a natural antibody it should be noted (Table III) that with increasing age there is an increasing ability to neutralize viruses of poliomyelitis. Thus, 70% of the cows (7-14 yrs), 27% of steers (1-2 yrs) and none of the calves (3-4½ months) had neutralizing antibodies in dilutions of 1:10 or higher. This increase with age is the typical pattern observed in antibody development. Table VI illustrates that this neutralizing activity is

TABLE IV. Titers of Antibodies in 20 Cow Sera against Poliomyelitis Viruses, Types 1, 2 and 3.

Cow sera	Titers of sera* Type 1	Type 2	Type 3
1	0	10	0
2	10	10	0
3	50	10	0
4	0	250	0
5	10	50	0
6	10	0	10
7	50	10	50
8	0	0	10
9	10	10	0
10	0	10	0
11	50	10	0
12	0	50	0
13	10	50	10
14	0	10	0
15-20	0	0	0
Total No. positive	8	12	4

* Expressed as reciprocal of actual dilution.

TABLE V. Titers of Antibodies in Steer Sera against Poliomyelitis Viruses, Types 1, 2 and 3.

Steer sera	Titers of sera* Type 1	Type 2	Type 3
1	0	250	0
2	0	50	0
3	0	250	0
4	0	250	0
5	0	250	0
6	10	0	0
7-22	0	0	0
Total positive	1	5	0

* Expressed as reciprocal of actual dilution.

TABLE VI. Neutralizing Activity of Pooled, Positive Animal Sera and the Resuspended Globulin Fraction against Types 1, 2 and 3 Polioviruses.

Animal sera, pooled	Neutralizing activity: Before globulin precipitation, Type 1	Type 2	Type 3	Resuspended globulin fraction, Type 1	Type 2	Type 3
Cow	+	+	+	+	+	+
Steer	+	+	—	+	+	—
Horse	+	+	—	+	+	—
Hog	+	+	+	—	+	—
Chicken	+	+	—	—	—	—

(+) Neutralization. (—) No neutralization.

retained in the precipitated globulin fraction of sera.

Though extensive studies were done with bovine sera, preliminary data has been included on neutralizing activity of horse, hog and chicken sera (Table VII). It will be noted that both horse and hog sera were often able to neutralize in high dilutions, while chicken sera were able to neutralize only in low dilutions. These limited studies do suggest that sera of horses and hogs also possess specific neutralizing antibodies to viruses of poliomyelitis.

Viral Isolation Studies. One possible interpretation of the antibodies observed (see discussion) is the assumption that cows may be infected with all three types of poliovirus. If this assumption is valid, it should be possible to isolate the virus of poliomyelitis from the bovine species. Preliminary isolation studies have been carried out and no viruses were isolated in HeLa cells which were neutralized by types 1, 2 or 3 poliomyelitis antibody. These studies were carried out with materials collected from cows, steers and calves; 470 spinal cords, 330 spleens, 310 lymph nodes and 410 feces specimens were studied as indicated under Methods.

TABLE VII. Neutralizing Activity Exhibited by Horse, Hog, and Chicken Sera against Types 1, 2 and 3 Polioviruses.

Animal	No. of animals	No. of animals positive	Antipoliovirus titer and type distribution of positive sera*: 1:10 Type 1	2	3	1:50 Type 1	2	3	1:250 Type 1	2	3
Horse	28	9	3	3	0	2	3	0	0	0	0
Hog	19	6	1	2	1	1	0	0	0	1	0
Chicken	29	2	1	1	0	0	0	0	0	0	0

* A single positive serum specimen may have contributed 1, 2 or 3 positive reactions depending on its ability to neutralize 1, 2 or 3 types of poliomyelitis virus.

Discussion. Our data indicate that neutralizing substances in bovine species are true antibodies for the reasons summarized below. 1. Antibody patterns in cows are highly specific, *e.g.*, some cows have no antibody, some have antibody to types 1 and 2, some to types 1 and 3, others to types 2 and 3, and still others have antibody to types 1, 2 and 3. This high degree of specificity, by definition rules out any non-specific neutralizing substance. 2. These are not natural antibodies for in addition to reasons stated above they are absent in young animals, increasing with age, and are not present in all adults of the species. In addition, titers are frequently high (1:250 dilution of serum neutralizes 100 TCD of virus). 3. Neutralizing substances are not inactivated by heating at 56°C for 30 minutes and are found in the globulin fraction of the serum.

It is therefore concluded that the neutralizing substances in cow sera are antibodies specific for polioviruses. It is important to note that these are antibodies *against* viruses of poliomyelitis and are not necessarily a result of infection with human strains of poliovirus.

While this study was in progress it was found that Dr. Albert Sabin (personal communication) was also carrying out a somewhat similar study(5). Dr. Sabin's data also indicate that neutralizing substances present in bovine sera are specific antibodies.

What is the origin of the antigenic stimulus? There are two possibilities: (1) These are antibodies resulting from subclinical infection of cows with human strains of poliovirus, with the implication that viruses of poliomyelitis exist and multiply in areas outside of man. (2). These antibodies arose not from the antigenic stimulus of human poliomyelitis strains but rather from viruses of non-human origin that are serologically related to human viruses. It is obvious that

such strains, if isolated, may be important as immunizing agents against poliovirus in humans. There is little experimental data to help us choose between these alternatives and little will be gained by further discussion of the possibilities since proof in either case requires the isolation from animals of the specific infectious agent.

Summary. (1) The sera of cows and steers were shown to possess neutralizing substances in high titer against the viruses of poliomyelitis. These have been shown to be specific antibodies against the viruses of poliomyelitis. (2) The sera of horses, hogs and chickens also possessed modest neutralizing activity against the viruses of poliomyelitis. (3) The sera of dogs, cats, calves and lambs do not possess neutralizing activity against the viruses of poliomyelitis. (4) Attempts to isolate a virus from the bovine species that might explain the origin of these antibodies were unsuccessful. The antigenic stimulus giving rise to antibodies against the viruses of poliomyelitis remains unknown.

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Myasthenia gravis. I. Importance of Potassium in Inhibitory Action of Normal and Myasthenic Thymus Extracts.* (22110)

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To investigate the concept that myasthenia gravis is due to a circulating blocking agent possibly elaborated by the thymus (McEachern)(1) several investigators(2-5) have tested thymus extracts on nerve-muscle preparations from various animals.

The present study was undertaken to repeat Dr. Wilson's experiments with the eventual objective of isolating the active blocking substance. The isolated frog sartorius nerve-muscle preparation, which had been used successfully by Wilson and Stoner(6) to detect the blocking activity of serum from myasthenic patients, was used in this investigation rather than the rat phrenic nerve-diaphragm preparation.

Methods. A. Isolated Frog Sartorius Preparation: Frogs were pithed and the sartorius muscle excised with a small block of underlying muscle to support the motor nerve. Frog Ringer, containing 6.5 g NaCl, 0.14 g KCl, 0.12 g $CaCl_2$, 0.2 g $NaHCO_3$ and 0.01 g NaH_2PO_4/l, bathed the muscle during dissection. After excision, the muscle was placed at normal resting length (5 to 10 g tension) in a plastic chamber of 2 ml capacity, provided with two sets of silver electrodes to permit independent direct and indirect stimulation of the muscle and nerve respectively. The small size of this chamber is of considerable advantage when only small volumes of test solution are available. The mechanical myogram was obtained by a transducer, consisting of an aluminum bar (6.5 x 1.2 x 0.015 cm) on either side of which was cemented a strain gage element.† The myogram was recorded with a Sanborn strain gage recorder (Model 127) fed from a Sanborn strain gage amplifier (Model 140). This arrangement permitted recording of the isometric myogram. All experiments were performed at 23 to 25°C. Thresholds for nerve and muscle excitability were recorded at beginning of each experiment. After 15 minutes equilibra-

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† Baldwin-Lima-Hamilton Corp., Type A7.