Toxoplasmosis II. Studies of Animal Sera Using Complement-Fixation Tests

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RESUME

Une étude sérologique fut faite chez des cobayes, un mouton, un veau, une chèvre et deux porcs expérimentalement infectés avec l'agent de la toxoplasmose. L'épreuve directe de la fixation du complément a décelé les anticorps dans les sérums de cobaye, de chèvre et de mouton. L'épreuve modifiée de la fixation du complément, dans laquelle une fraction de sérum bovin normal est ajoutée au complément, fut nécessaire pour éprouver les sérums bovins.

Avec les sérums de porc, les meilleures réactions furent obtenues avec l'épreuve indirecte de la fixation du complément. Des réactions bien marquées mais peu élevées furent obtenues avec l'épreuve directe après que l'activité pro-complémentaire des sérums de porc fut inactivée par acidification.

Des réactions allergiques furent obtenues chez ces animaux d'expérience mais l'activité de l'antigène devrait être améliorée avant que cette épreuve puisse être employée pour le diagnostic de la toxoplasmose chez les animaux.

SUMMARY

Serological studies were performed in guinea pigs, a sheep, calf, goat and two pigs experimentally infected with toxoplasmosis. The direct complement-fixation method was effective in detecting antibodies in guinea-pig, goat and sheep sera. The modified complement-fixation technique supplementing complement with normal bovine serum fraction, was required when testing bovine serum. With swine sera best reactions occurred in the indirect complement-fixation test and definite but low grade reactions were produced in the direct test after pro-complementary activity was removed by pH treatment of the sera.

Allergic skin reactions were produced in the experimental animals but improvement in the antigen is necessary before the test could be used generally in the field as a diagnostic method for animal toxoplasmosis.

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Toxoplasma infection has been recognized in a wide variety of animals and birds as well as in man in many parts of the world. The literature was well reviewed by Wolf (1).

The most important clinical signs of infection are encephalitis, febrile exanthema, pneumonitis and enterocolitis. The species and age of the host and the tissues affected determine the character of the clinical signs. Laboratory procedures are required to assist in diagnosis.

Positive identification achieved by inoculation of mice or guinea pigs with the suspected infective material and subsequent examination of peritoneal exudate is a cumbersome and time consuming method. Presumptive diagnosis may be made by other methods such as histopathological studies, the dye test, complement-fixation (CF) ' test, skin test and fluorescein-labelled antibody technique. The CF test as suggested by Warren and Sabin (2) for the testing of human sera is reported to frequently give negative results in the detection of antibodies in sera from various species of animals such as sheep, cattle, swine and chickens (3, 4). The dye test (5) is said to give a greater percentage of reactions than the CF test and antibodies appear to be detectable for long periods after infection. The skin test, developed by Frenkel (6) for human infection, has been found less sensitive than the dye test.

The CF test was chosen for our studies as possibly a better indicator of active infection than other methods and is a procedure employed routinely for other infections in many serological laboratories.

Materials and Methods

Infective Agent

Toxoplasma gondii, RH strain, was obtained from Dr. N. A. Fish, Ontario Veterinary College, Guelph, Ontario. It was maintained by transfer in adult mice and young rats. Mice were injected intraperitoneally at three or four day intervals with 0.2 ml. of a 1:10 dilution of infected mouse peritoneal exudate. Rats were inoculated intracranially once a month with 0.15 ml. of similar material to serve as a reservoir of virulent material.

Immune Sera

Guinea pigs: These animals were selected because, in general, their serum antibodies readily fix complement and exhibit little tendency toward anticomplementary effect. Adult guinea pigs were infected by intraperitoneal inoculation with 0.25 ml. of undiluted peritoneal exudate collected from infected guinea pigs at height of temperature. Injections were repeated at weekly intervals for a total of five inoculations. To reduce the number of deaths because of toxoplasmosis, sulphonamides were administered at the time of the first injection. Serum samples were obtained before the first and again three weeks after the last injection. Guinea pigs showing good CF serum titres were bled out and the sera stored frozen.

Large animals: A three month old calf, an adult ewe, a mature goat and two 61 day old specific pathogen free piglets were used. The calf, goat and ewe received 0.1 ml. of infected mouse peritoneal exudate administered intraperitoneally. The piglets were fed similar amounts of this material. Blood was collected before exposure and again at regular intervals for 78 days afterward. Sera were stored frozen.

Complement-Fixation Tests

Antigen: Antigen was prepared by a modification of Nobuto's method (3) as follows. Ten day old embryonated chicken eggs were inoculated on the chorioallantoic membranes with 0.2 ml. of a 1:10 dilution in normal saline of infective mouse peritoneal exudate. Membranes and fluid were harvested five days post inoculation and combined in a ratio of approximately 1 volume of membrane material to three volumes of fluid. Merthiolate was added to a final concentration of 1:10,000 and the material was frozen. When required the suspension was thawed and frozen three times, ground in a Ten Broeck grinder and sonic vibrated 10 minutes in a Raytheon 10 KC magnetostrictive oscillator. Coarse particles were removed by centrifugation at 2000 g. for 10 minutes. The supernatant fluid was dispensed in 1.0 to 2.0 ml. quantities and stored frozen for use as antigen.

Direct CF test: Sera of all experimental animals were tested by the direct CF test in serial dilutions ranging from 1:5 to 1:160. Three corrected 50 per cent haemolytic units of complement were used. (A correction factor of 1.5 was employed to compensate for deterioration of complement during the 18 hours incubation at 9°C.). All reagents of the test were added in 0.1 ml. The period of primary incubation for fixation was 18 hours at 9°C., that of secondary incubation after the addition of 0.2 ml. of a 2.5 per cent suspension of maximally sensitized sheep red cells, was 30 minutes at 37°C. The serum titre was recorded as the highest dilution with which 50 per cent haemolysis or less was obtained in the presence of the antigen. A variation of the test using two haemolytic units of complement was employed in retests of the swine sera. Swine sera were further tested after treatment by two methods to remove "procomplementary" activity. In the first method the sera were acidified by a technique previously described by Boulanger (7, 8). The pH of the sera was reduced to 4.2 and after standing for 18 hours at 9°C. was restored to 7.4. The sera were inactivated for 30 minutes at 56°C. and tested. The second method involved addition of phenol to the sera to a concentration of 0.5 per cent. The sera were inactivated at 56°C. for 30 minutes and tested 6 hours and 18 hours after phenolization.

Modified direct complement-fixation test: This test was performed on sera of the calf, goat and sheep. As previously described by Boulanger (9) the technique was the same as for the direct method with the exception that the veronal buffer used in making the complement dilution contained five per cent normal unheated bovine serum fraction as supplementing factor. The fraction was prepared by dialysing fresh normal cow serum against phosphate buffer pH 6.2 for 2 hours in the cold. The precipitate obtained was washed three times with

phosphate buffer then restored to the original serum volume with veronal buffer. The fraction was frozen until used. For use, the fraction was diluted 1:20 in buffer containing five per cent heated guinea-pig serum and this mixture was employed as diluent for the complement.

Indirect complement-fixation test: This method was employed in testing swine and calf sera using an adaptation of the method described by Rice (10) for the *S. pullorum* test. Dilutions of inactivated test sera were mixed in 0.1 ml. amounts with 0.05 ml. of the antigen and incubated at 9°C. for 18 hours. The antigen was employed in the highest dilution of which 0.05 ml. had given complete fixation of three 50 per cent haemolytic units of complement in the antigen titration. Following the first incubation, 0.1 ml. of a complement dilution containing three corrected haemolytic units was added followed by 0.05 ml. of inactivated guinea-pig or goat immune serum containing an excess of toxoplasma antibody. The tubes were shaken and the test incubated a further 18 hours at 9°C. Finally 0.2 ml. of a 2.5 per cent suspension of maximally sensitized sheep red cells were added and the test was incubated at 37°C. in a water bath for 30 minutes. The serum titre was recorded as the highest dilution with which 50 per cent haemolysis or more was obtained in the presence of antigen.

Conglutination Test

Conglutinating complement-fixation tests were performed on sera of the calf. The technique employed was that used by Rice *et al* (11, 12) in testing bovine serum for brucellosis. Antigens used were the CF antigen already described and one prepared from infected mouse peritoneal exudate as follows. Sediment from the exudate was washed three times with saline, resuspended to original volume in distilled water, frozen and thawed eight times in a dry ice-alcohol bath and centrifuged. Approximately 0.5 ml. of this sediment was resuspended in 10 ml. of cold saline and sonic vibrated for 15 minutes. Merthiolate to 1:10,000 was added and the antigen was stored frozen until used.

Dilutions of the sera, two or three conglutinative units of horse complement and antigen were mixed in 0.1 ml. amounts and incubated together for one hour at room temperature. After the period of primary incubation 0.2 ml. of sensitized sheep red cells were added and the test was incubated 15 minutes at 37°C in a water bath. Sensitization of the sheep red cells was accomplished by mixing equal parts of a one per cent suspension of erythrocytes with an equal part of the sensitizing solution. This consisted of equal parts of an optimum amount of heated bovine serum to supply conglutinin and rabbit amboceptor to augment the natural antibody of bovine serum. The rabbit amboceptor was diluted to one fifth the concentration used in the haemolytic CF test. Following secondary incubation the tubes were centrifuged at approximately 200 x.g. for 3-4 minutes and read by the re-suspension technique. Titres were expressed as the highest serum dilution showing 2+ agglutination.

Dye Test

Only sera from the guinea pigs were tested by the methylene-blue dye test performed as described by Frenkel and Jacobs (13).

Skin Test

All experimental animals were tested at intervals following exposure by the intradermal injection of 0.2 ml. of skin test antigen and readings were made after 24, 48, 72 and 96 hours. Two lots of skin test antigen were prepared by Nobuto's method (14) from *Toxoplasma gondii* recovered from peritoneal exudate of infected mice. Comparative tests were made with a skin test antigen obtained from Nobuto.

Results

CF Antigen Titrations

Four lots of CF antigen were prepared from chorioallantoic membrane. Titrations of these with positive guinea-pig sera revealed titres in the order of 1:64 or slightly higher with the exception of one batch which reacted only to the 1:8 dilution. In this instance fluctuations in temperature during incubation of the infected embryonated eggs was thought responsible for the lower antigenicity. One lot of dessicated antigen kindly supplied by Dr. Nobuto of the National Veterinary Assay Laboratory, Tokyo, was titrated and found reactive to the 1:16 dilution.

Variation in reactivity of antigens was observed in titrations made with various lots of antiserum. A slight drop in titre

TABLE I — Results of CF tests of goat, sheep and calf sera collected before and at intervals after injection with T. gondii.

Test employed	Titre[1]								
	PRE	2 wk.	3 wk.	4 wk.	6 wk.	8 wk.	10 wk.	12 wk.	4 mo.
Goat									
Direct CF	10[2]	80	160	>160	160	80	80	80	80
Sheep									
Direct CF	40	80	80	80	40	40	40	80	40
Modified Direct CF	20	>160	160	40	80	80	80	80	80
Calf									
Direct CF	< 5	10	20	20	20	10	10	10	5
Modified Direct CF	20[2]	160	160	160	160	80	160	160	80

[1]Titre is expressed as the reciprocal of the highest serum dilution giving 50 per cent haemolysis or less.
[2]Incomplete fixation.

occurred with ageing necessitating retitration of antigen at intervals of two or three months.

GUINEA-PIG SERA

Sera collected from a group of nine guinea pigs experimentally infected for production of positive control serum were tested by the direct CF method. An excess of antigen, i.e. four units, was employed in the test. While a serum titre of 1:80 was obtained with one animal the remainder ranged from 1:60 to 1:640. A further group of nine guinea pigs infected eleven months later developed serological titres of 1:80 to 1:160. Pre-infection sera gave negative reactions and none of the pre and post-infection sera were anticomplementary or reacted with normal egg control antigen.

A pool of the guinea-pig sera was used in tests to compare our antigen with that obtained from Nobuto. Dessicated serum also obtained from Nobuto was reconstituted and used for comparison with our sera. In tests performed with four units of Nobuto's antigen the titre of Nobuto's

TABLE II — Results of direct CF tests using untreated, acidified and phenolized sera and of indirect CF tests performed on swine sera collected before and after exposure to T. gondii.

Time Sera Collected	Direct CF Titres[1] (Untreated) Serum		Direct CF Titres[1] (Acidified) Serum		Direct CF Titres[1] (Phenolized) Serum		Indirect CF Titres[2]	
	Pig 46	Pig 47	Pig 46	Pig 47	Pig 46	Pig 47	Pig 46	Pig 47
Pre	—	—	—	—	—	—	10[4]	10[4]
2 weeks	40[3]	—	10	5	20	—	80	80
3 weeks	10	—	10	10	10	5	40	10
4 weeks	40	—	20	20	10	5	40	20
6 weeks	40	—	20	10	10	10	40	20
8 weeks	10	—	10	20	10	10	40	40
10 weeks	10	10	10	20	10	10	20	40
12 weeks	ND	20	10	10	10	10	20	20
16 weeks	< 5	10	5	20	5	10	40	40

[1]Titre is expressed as the reciprocal of the highest serum dilution giving 50 per cent haemolysis or less.
[2]Titre is expressed as the reciprocal of the highest serum dilution giving 50 per cent haemolysis or more.
[3]Incomplete fixation.
[4]Incomplete inhibition.

serum was 1:80 and of ours was 1:40. In comparative tests with our antigen the titres were 1:80 and 1:160 respectively. The reconstituted Japanese serum was slightly anticomplementary.

Goat serum

The results obtained by the direct CF method with sera collected from the goat before and at intervals after experimental inoculation are given in Table I. The pre-inoculation serum showed a partial reaction in the 1:10 dilution. The titre increased to 1:80 two weeks after exposure. A peak titre in excess of the 1:160 dilution was reached four weeks after inoculation but dropped to 1:80 after a further month and remained at that level until the last bleeding four months post-inoculation. There was little tendency for the goat serum to show anticomplementary activity nor to react with the normal egg antigen.

In view of the reactions obtained using the direct CF test it was felt unnecessary to investigate the more cumbersome modified technique.

Sheep serum

Pre and post-inoculation sheep sera were tested by the direct and modified direct CF methods. Results are compared in Table I. The high titres obtained with pre-inoculation serum in both tests together with the rapid rise to peak post-inoculation levels suggested that the animal had been infected previously. This hypothesis is further supported by temperature response of the various animals as illustrated in figure 1. Whereas the goat, calf and swine developed plateau shaped temperature reactions commencing on the fourth day after inoculation and lasting for three to five days, the sheep temperature rise was of one day duration and occurred the day following inoculation. Reactions in the direct CF test ranged from a peak of 1:80 two weeks after inoculation down to 1:40 after four months. Generally, reactions in the modified test were one dilution higher. Control tests using normal egg antigen yielded trace reactions with six of the sera when the direct test was used and with one in the modified test. As with the goat serum it was found unnecessary to use the modified test. However, inactivation of the sera at 60°C. reduced the anticomplementary effect of sheep serum.

Calf Serum

Direct CF tests of calf sera yielded low titres as shown in Table I. A peak titre of 1:20 was observed from the third to the sixth week post inoculation. This dropped to 1:5 by the fourth month. When the modified direct CF test was applied serological titres were three to four fold higher. However, partial reactions were obtained in the 1:5 dilution using normal egg antigen. Preliminary tests of the calf sera made by the indirect CF method showed little inhibition of fixation.

Swine Serum

Table II presents the results obtained in four of the tests conducted on the porcine sera. In the direct CF test sera from pig 46 gave only partial fixation up to the 1:40 dilution during the first two to six weeks after exposure. Sera from pig 47 reacted similarly to the 1:20 dilution 12 weeks post exposure. However, because the reactions were atypical confidence could not be placed in these results. The reliability of the results was not increased when two units of complement were employed instead of three.

Repetition of the direct test employing pH treated sera gave titres in the 1:10 to 1:20 range. While titres were low com-

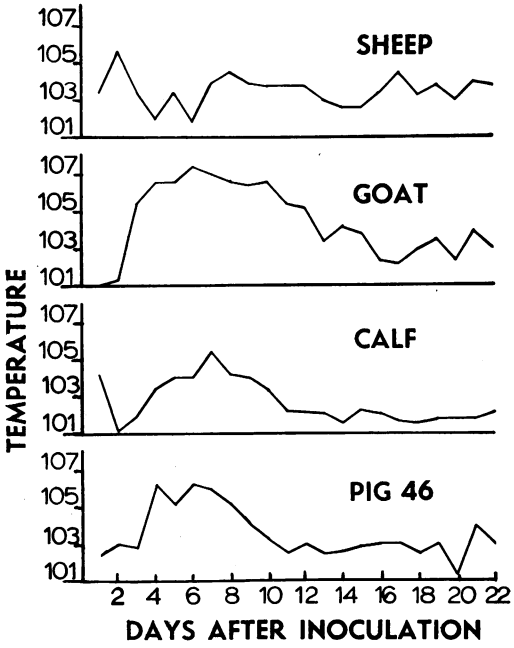

Figure 1: Temperature response of experimental animals following exposure to T. gondii

plete fixation was obtained in these dilutions. Control tests using normal egg antigen were negative and the sera were not anticomplementary. The direct CF test employing phenol treated serum yielded slightly lower titres and fixation was incomplete.

In preliminary trials with the indirect CF method it was found that immune sera of either the guinea pigs or goat were usable as a source of antibodies for the second stage of the test but goat serum was preferred because larger quantities were readily available. The titres of the post-inoculation swine sera ranged from 1:20 to 1:80. The sera from the pre-inoculation bleedings showed partial inhibition in the 1:10 dilution.

Conglutination Tests

In view of the difficulties in standardizing the modified direct CF test for the detection of toxoplasma antibodies in cattle serum the conglutination method was investigated. Both the egg CF antigen and the mouse peritoneal exudate antigen failed to react with the calf sera. These findings were unexpected in view of the sensitivity of the technique when used with bacterial antigens such as *Br. abortus*. A modification of the method was tried incorporating fresh unheated normal cattle serum to the complement to supply a possible supplementing factor as required in the modified CF test. This did not improve the reaction.

Methylene Blue Dye Test

The standardization of the method was made on guinea-pig sera known to have a high antibody content as determined by the CF test. Only a quantitative difference was obtained between the normal and immune guinea-pig sera. For example, with the normal sera at most 80 per cent of the organisms were stained whereas with the positive sera complete inhibition was never observed. There always remained at least 25 per cent of the organisms which absorbed the stain. Often the difference in inhibition between the normal and positive sera was less pronounced. Furthermore, considerable difficulty was experienced in obtaining suitable human accessory factor. These considerations together with the danger and labor involved in maintaining the organism in mice and the microscopic reading of the test suggested it was impractical for testing large numbers of sera routinely.

Skin Test

Skin tests performed with antigen lot 1 on the sheep, goat, calf and swine before administration of toxoplasma and again 4, 6 and 10 weeks after were all negative. However, the sheep showed a slight greyish discoloration at the point of inoculation. Various degrees of reaction were observed in a final test at 16 weeks after exposure using antigen lots 1 and 2 and Nobuto's antigen. These reactions in the sheep and both pigs were characterized by redness and swelling and were at a peak at the 48 hour reading. The optimum reaction was seen in the goat at 72 hours and in the calf at 96 hours. In all of these tests antigen lot 2 was most reactive.

Two guinea pigs also tested with antigen lot 1 five weeks after exposure failed to react but gave definite reactions at the eleventh week with the same product. The three lots of antigen were used at the 21st week and all elicited a response. Antigen lot 2 was again most reactive.

These preliminary results obtained with the allergic test indicate that the skin sensitivity appeared a relatively long time after exposure and was of low intensity. Considerable additional work would be necessary before such a method could be relied upon for routine diagnosis.

Discussion

In the diagnosis of any infection a simple, sensitive and reliable in vitro test is desirable. In the diagnosis of toxoplasmosis the dye test, in our hands, did not fully meet these requirements. Among other reasons difficulty was encountered in securing a satisfactory donor for the human serum accessory factor required in the test. In addition the necessity of constantly maintaining infection in mice as a source of antigen is a hazard to laboratory workers and is time consuming. The reading of the test requires counting of large numbers of organisms microscopically and at best the results are not clear cut.

An allergic test which could be performed in the field would be a practical method of diagnosis. The low degree of reactions obtained with the skin test antigen and their late appearance after exposure as observed in our preliminary trials suggests that a

more reactive antigen is needed before the method could be adopted for general use.

Serological diagnosis by the direct CF test presents no difficulty when testing for guinea-pig, goat and sheep serum antibodies. The direct test was not effective for detection of antibodies in calf serum as previously pointed out by Nobuto (3). A modified direct CF test obviated this difficulty although the normal serum supplementing factor must be prepared and standardized carefully. In contrast with the results reported by Nobuto (3) the indirect CF method did not detect the cattle serum antibodies. This may be explained by the fact that our experimental animals received one exposure and developed no clinical manifestations other than temperature response.

The detection of swine serum antibodies is possible by the indirect test or by the direct test employing pH treated serum. However, these tests are more complex than the routine direct method and require careful standardization.

ACKNOWLEDGEMENT

The authors wish to acknowledge the technical assistance of Messrs. G. C. Gollain, L. Gauthier, W. A. Boyd and G. A. Casey.

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A comparison of experimental anaphylactic shock in guinea pigs with naturally occurring oedema disease and haemorrhagic gastroenteritis in pigs

By subjecting guinea pigs to protracted anaphylactic shock, using egg albumen and *E. coli* extracts, symptoms and lesions were produced which resembled those of oedema disease and haemorrhagic gastritis in pigs. *E. coli* polysaccharide introduced into the stomach was absorbed rapidly and caused anaphylactic shock in sensitized animals. Healthy guinea pigs were to some extent hypersensitive to *E. coli* extracts. The anti-histamine mepyramine maleate prevented death from acute anaphylactic shock, but was not effective against protracted anaphylaxis.

Lymphocytes, plasma cells and eosinophil leucoytes, the predominant cell types associated with the lesions of oedema disease and haemorrhagic gastroenteritis in pigs, were also seen in the lesions of anaphylactic shock in guinea pigs. Plasma cells containing eosinophilic granules were most numerous in guinea pigs in association with the more protracted symptoms. These cells were observed in larger numbers from pigs with haemorrhagic gastroenteritis than with oedema disease and were very numerous in lesions of necrotic enteritis. The presence of these cells along with advanced degenerative changes in lymphoid tissue suggests that necrotic enteritis may also develop as a result of an anaphylactic reaction. The occurrence of haemorrhage and old-

er necrotic lesions in the same pig suggests that anaphylactic reactions may be repeated in an individual animal.

It is postulated that the symptoms and lesions of oedema disease and haemorrhagic gastroenteritis are the result of anaphylactic shock reaction rather than a direct toxic effect following the absorption of *E. coli* polysaccharide.

Thomlinson, J. R. and Buxton, A. Res. Vet. Sci. 3: 186-202, 1962.

The relationship between milking machine practice and bovine mastitis

The possible relationships between the milking machine and mastitis were divided into two categories, those connected with the machine as a vector of disease organisms and those related to its traumatic effects.

One of the worst components of the machine for harboring organisms was the teat cup. Due to perishing of the rubber liner a refuge for pathogens was established. Normal cleaning procedures were not effective in removing these organisms. The regular changing of rubberware and the use of non-perishable synthetics were logical steps in the control of infection spread. Satisfactory dipping of teat cups after milking was difficult and the use of hot water (165-170°F.) plus a soluble disinfectant agent was suggested. Chlorexidine (hibitane) appeared to be one of the most efficient. There was often transfer of milk from infected to uninfected quarters during milking. Stress was placed on good "intercow" disinfection, both of udder and milking machine.

As a traumatic agent attention should be paid to proper vacuum level. High vacuum resulted in teat erosions; erosion of the teat canal, cyanosis of teats and oedema of tips. High pulsation rates also appeared to be an undesirable factor.

Whittlestone concluded by stating that the first essential of good machine milking techniques was good hygiene. In addition cows should be stimulated so that milk was effectively let down. As soon as the rate of milk flow fell to one-half pound per minute the teat cups should be removed. The antiseptic of choice was chlorhexidine which should be used as a teat dip after milking as well as a teat cup disinfectant. A strip cup should also be used to stimulate milk flow and to give the milker some sign of incipient mastitis.

Whittlestone, W. G. Aust. Vet. J. 38:114-118, 1962.

Observations on paw-raising and sympathy lameness in the dog.

Paw-raising in the dog is seen as a normal response to social stimuli or may be observed as an abnormal psychological manifestation where no clinico-pathological lesions can be found. Distinction must be drawn between normal and abnormal behaviour. Normal forepaw raising is first seen in response to licking by the bitch. This activity pattern persists and is especially evident at the prepubertal and pubertal stages of development. Forepaw raising may be following by urination in the case of submissive dogs. Highly nervous strains have an inherited diathesis to this condition. Paw-raising, recumbency, urination, defaecation and increasing nervous tension are common in fear-biting dogs being examined by strangers. Paw-raising and sympathy lameness is common in petted dogs. If trodden on by the owner early conditioning to reward sympathy following a mild foot injury causes the dog to limp to the slightest traumatic provocation, even though the site of injury may be remote from the apparent lameness. Often after traumatic nerve lesions, where reflex examination indicates good recovery, the patient may refuse to use the affected limb. In the clinical differential diagnosis, full reflex examination should be carried out where it is important to differentiate between behaviour caused by organic lesions — in which the behaviour is "normal" — and behaviour due to maladaptation where no lesions are observed.

Fox, M. W.
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