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DETERMINATION OF SPECIFIC ANTI-LEPTOSPIRAL IMMUNOGLOBULINS M AND G IN SERA OF EXPERIMENTALLY INFECTED DOGS BY SOLID-PHASE ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

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The development and evaluation of an enzyme-linked immunosorbent assay (ELISA) to detect specific anti-leptospiral IgM and IgG in sera of dogs experimentally infected with *Leptospira interrogans* serotype canicola are reported. In all dogs specific anti-leptospiral IgM was detected from the second half of the first week after infection, the maximum being attained during the second week. Subsequently the IgM titre gradually decreased. Specific anti-leptospiral IgG was detected later and increased gradually to reach almost the same level as the IgM titre after two to three months.

During the initial stage of the infection, when the microscopic agglutination titre was still negative or very low, a high IgM titre was accompanied by a negative or very low IgG titre in every case. After the initial stage a substantial IgG titre was also detectable. It is suggested that the test is suitable for serodiagnostic purposes, particularly for the diagnosis of a current infection in an individual.

INTRODUCTION

The microscopic agglutination test (MAT) continues to be the recognized standard reference test for the serodiagnosis of leptospirosis. Though specific, the MAT is not very sensitive. High titres are often not observed until the second week after the onset of the disease (Wolff, 1954; Turner, 1968). Low (doubtful) antibody titres, which are a common finding, are a serious problem in diagnosis. Frequently only a single serum sample is available so that a possible increase in titre cannot be detected. Administration of large doses of antibiotics at an early stage of the disease may cause the MAT titre to remain at a low level (Wolff, 1954; Babudieri, 1961; Turner, 1968). Vaccination usually results in the production of only low MAT titres.

The question then remains whether a low MAT titre is the result of (a) the initial stage of antibody production in response to a current infection,

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(b) a previous infection or (c) a vaccination. When the MAT or ELISA are used, it is not possible to discriminate with certainty between these possibilities (Hartman et al., in press).

In various animal species and in man, IgM is the first immunoglobulin that can be detected following antigenic stimulation. It is followed by the appearance of IgG antibodies some time later. As a rule, the IgG response continues for a more prolonged period than does the IgM response which usually decreases within a few weeks (Pike, 1967; Crawford, 1972; Hood et al., 1978). If the amount of IgM and IgG directed against leptospires is measured, a better insight may be gained into the course of antibody production following infection with leptospires or vaccination against leptospirosis.

Is has been established in man and in a number of animals that not alone IgM, but, although to a lesser extent, also IgG will cause agglutination of particle antigen (e.g. leptospires) (Pike, 1967; Crawford, 1972; Bey and Johnson, 1978a; Adler et al., 1980) so that the MAT titre in itself does not allow conclusions to be drawn about the class of antibody concerned and, consequently, about the stage of the disease. The ELISA technique has been used to identify the specific classes of immunoglobulin (IgM or IgG) which are produced against various pathogenic micro-organisms (Voller et al., 1978; Adler et al., 1980; Kortbeek-Jacobs and van Houten, 1982). In the present paper results are reported of the detection of specific anti-leptospiral IgM and IgG in the serum of dogs experimentally infected with Leptospira interrogans serotype canicola using an ELISA technique developed by the present authors.

MATERIALS AND METHODS

Dogs

Five 1- to 2-year-old seronegative (MAT) dogs of either sex were experimentally infected by intraperitoneal inoculation of 5 ml of a hamster liver suspension containing hamster-virulent Leptospira interrogans serotype canicola leptospires as described by Hartman et al. (in press).

Serum samples

Serial serum samples were collected from each dog before and after experimental infection and stored at -70^{0}C until use. Control sera were obtained from seronegative (MAT) beagles.

Microscopic agglutination test (MAT)

The MAT (Wolff, 1954; Turner, 1968) was carried out in Cooke Microtiter plates as described previously (Hartman et al., in press).

ELISA

Polystyrene microtitre plates (Cooke Dynatech, Microelisa) were coated overnight at room temperature with outer envelope (OE) antigen of Leptospira interrogans serotype canicola strain Hond Utrecht IV leptospires (2 μg/ml of protein) as described by Hartman et al. (in press).

Test procedure

The antigen-coated plates were washed thoroughly three times with a total amount of 2 l of tap water containing 0.05% (v/v) of Tween 20. Serial two-fold dilutions of serum, starting with 1 in 20, were made in quantities of 100 μl in PBS 7.4 with 0.1% Tween 20 and 1% egg albumin (Difco 0255-15). The plates were incubated for 2 h at 37°C. After thoroughly washing three times with 2 l of tap water-Tween 20, 100 μl of a 1 in 1000 dilution of heavy-chain specific rabbit anti-canine IgM or rabbit anti-canine IgG in PBS-Tween 20-egg albumin were added. The plates were incubated for 1 h at 37°C and washed three times as described above. Then 100 μl of goat anti-rabbit IgG (H + L) horse-radish peroxidase conjugate (Miles Laboratories), diluted 1 in 1000 to 1 in 2000 in PBS-Tween 20-egg albumin, were added to each well and the plates were incubated for 1 h at 37°C.

The optimum conjugate dilution for each batch was determined by chequerboard titration with positive and negative sera.

Finally, the plates having been washed three times with tap water-Tween 20, 100 μl of substrate (5-amino-2-hydroxybenzoic acid) were added. This substrate and its enzymic degradation product are partially insoluble and therefore only acceptable for visually read ELISA tests (Voller et al., 1978). The final result of the test was assessed visually. The titre is expressed as the reciprocal of the highest serum dilution producing a clearly perceptible coloured reaction product. Controls of antigen, conjugate and substrate were included in the test.

Preparation of specific rabbit anti-dog immunoglobulin sera

Canine IgM was purified by successive delipidation, ammonium sulphate precipitation, dialysis against distilled water and gel filtration of the euglobulins on ACA 22 (LKB, Stockholm) (Goudswaard et al., 1978). Canine IgG was isolated from serum by gel-filtration and ion-exchange chromatography as described by Heddle and Rowley (1975).

Rabbits were immunized by multiple intramuscular as well as subcutaneous injection of 2-5 mg immunoglobulin emulsified with Freund's complete adjuvant (Difco). The rabbits were boosted three weeks later without Freund's complete adjuvant. The antisera to IgM and IgG were rendered specific to μ and γ heavy chains respectively by affinity chromatography on CNBr-activated Sepharose 4B (Pharmacia), to which the purified non-corresponding immunoglobulins were

coupled. The IgG fraction of the monospecific rabbit antisera was isolated by affinity chromatography on a Sepharose CL-4B column with protein A (Pharmacia) (Goudswaard et al., 1978). The specificity of the antisera was compared with monospecific antisera (Dr. J.P. Vaerman, Université Catholique de Louvain) and tested against pure IgM and IgG and whole dog serum by immunodiffusion, immunoelectrophoresis and the ELISA technique. Immunodiffusion and immunoelectrophoresis against pure canine IgM and IgG and whole dog serum did not reveal cross-reactions. In the ELISA with pure canine IgM as antigen, cross-reactions were not observed with rabbit anti-dogIgG serum. However, a slight cross-reaction was seen in the IgG-coated wells when incubated with rabbit anti-dogIgM serum. This slight cross-reaction was found to be caused by the goat anti-rabbit conjugate, as a similar reaction occurred in the IgG-coated wells incubated with the goat anti-rabbit conjugate alone. The goat anti-rabbit conjugate is apparently also bound to the canine IgG to some extent.

This minor non-specificity of the goat anti-rabbit conjugate was accepted as it did not interfere with the interpretation of the test and, moreover, the goat anti-rabbit conjugate was commercially available (Miles Laboratories). It could, however, be avoided by using horse-radish peroxidase conjugated rabbit anti-dog-IgM and -IgG sera.

The subclass specificity of the rabbit anti-dogIgG serum was not tested.

RESULTS

The results of the titration of the sera of one out of five experimentally infected dogs (dog no. 4) are shown in Table I. As the titration of the sera of the other four dogs gave essentially the same results, these are omitted.

The IgM ELISA showed some non-specific reactions in the lower dilutions (maximum 1:40) of the MAT negative control sera. IgM titres ≤ 40 should therefore be considered to be non-specific. Non-specific reactions were not observed with MAT negative control sera in the IgG ELISA so that IgG titres ≥ 20 may be regarded as specific.

All dogs produced both specific anti-leptospiral IgM and IgG. The production of specific IgM increased considerably in all dogs during the second half of the first week after infection. The maximum was attained by the end of the first week or during the second week after infection, and varied from 1280 to 5120. When the maximum had been reached, the IgM titre declined slowly. During the initial stage of the immune response to the leptospiral infection a positive reaction was detected with serum of three out of five dogs in the non-antigen coated control wells when incubated with the anti-dogIgM serum. This reaction was due to aspecificity of the initially produced IgM, which reacted not only with leptospiral antigen but also with canine IgG, egg albumin, bovine albumin, horse serum and lipopolysaccharide of *Escherichia coli*. In every case the titre

with the non-leptospiral antigen was lower than the titre with the leptospiral antigen and did not exceed 160.

The production of specific anti-leptospiral IgG started later and increased less rapidly than did the production of specific anti-leptospiral IgM. IgG titres $\geqslant 20$ were detected for the first time in four out of five dogs during the second week after infection. Maximum levels varying from 80 to 160 were not attained before the fourth week after infection. Reduction of the IgG titre did not start until approximately two months after infection. During the first two to three weeks after infection a marked difference between the amounts of IgM and IgG was detectable, whereas this difference disappeared during the subsequent stage. The IgM/IgG ratio (calculated as the ratio of the log 10 of the IgM and IgG titres) decreased in all dogs during the experimental period (maximum equal to or exceeding 2.39) and ultimately approximated a ratio≈1 (Table I). Both IgM and IgG continued to be detectable throughout the experimental period.

A titre $\geqslant 160$ was attained by the IgM ELISA on average 6.2 days earlier than it was by the MAT. Using Student's t-test for related observations this difference proved to be significant ($t=5.81$; $df=4$; $P<0.01$). During the initial stage after infection a positive IgM titre was observed by the ELISA in three out of five dogs, whereas the agglutination titre was still entirely negative.

TABLE I

Anti-leptospiral IgM and IgG detected by ELISA in serum of one out of five experimentally infected dogs (dog no. 4). The results are representative of all dogs under experiment.

day	MAT	IgM	IgG	IgM/IgG ratio
0	θ	40	θ	-
2	θ	θ	θ	-
4	θ	40	θ	-
7	θ	640	20	2.16
9	θ	1280	20	2.39
14	320	5120	80	1.95
24	320	1280	80	1.63
31	320	640	160	1.27
45	160	320	160	1.14
52	80	320	160	1.14
87	20	80	80	1
122	20	80	80	1

θ = <20

DISCUSSION

Various investigators have studied the classes of specific anti-leptospiral immunoglobulins in several animal species and in man. Both specific anti-leptospiral IgM and IgG have been detected in the sera of human individuals (Sulzer et al., 1975; Adler et al., 1980), cattle (Morris and Hussaini, 1974; Tripathy

et al., 1975; Bey and Johnson, 1978b), dogs (Bey and Johnson, 1978a), rabbits (Adler and Faine, 1978), and guinea pigs (Crawford, 1972). The anti-leptospiral activity of IgM and IgG has been demonstrated in immunoglobulin fractions of serum using the microscopic agglutination test (Crawford, 1972; Morris and Hussaini, 1974; Adler and Faine, 1978; Bey and Johnson, 1978a; Adler et al., 1980), the leptospiricidal assay (Bey and Johnson, 1978a, b) and the growth inhibition test (Tripathy et al., 1975). Selective inactivation of IgM agglutinins by treatment of sera with 2-mercaptoethanol (Crawford, 1974; Sulzer et al., 1975) or dithiothreitol (Morris and Hussaini, 1974; Fathally and Coghlan, 1980) has also been employed. The drawback to methods based on agglutination titres is that the titres do not become positive until a considerable time after infection and that only agglutinating antibodies are detected.

Specific anti-leptospiral IgM and IgG were demonstrated by Negi et al. (1971a, b) in bovine serum by a passive haemagglutination test in which specific anti-IgM and anti-IgG were also used. This method is to some extent comparable with the ELISA technique described in the present paper. The ELISA was used previously in identifying specific classes of immunoglobulin directed against various pathogenic micro-organisms in several animal species and in man (Voller et al., 1978; Adler et al., 1980; Kortbeek-Jacobs and van Houten, 1982). Following experimental infection of dogs with L. interrogans serotype canicola, specific anti-leptospiral IgM was already detectable by the end of the first week after infection using the ELISA technique, and the titre subsequently showed a rapid increase. On the other hand, specific anti-leptospiral IgG was not detectable before the second week after infection. The anti-leptospiral IgG titre increased less rapidly, attained a maximum level later and decreased later than the anti-leptospiral IgM titre. Graves and Faine (1970) also found that IgM continued to be detectable throughout the experimental period in rabbits infected with leptospires. Bey and Johnson (1978a) observed that IgM was also the predominant antibody during the first six weeks after leptospirosis vaccination of dogs, whereas the IgG class was the predominant antibody from weeks 6 through 52. During the subsequent course of the two years in which the dogs were studied, both Ig class antibodies were detectable in almost equal amounts. Bey and Johnson used the microscopic agglutination test and the leptospiricidal assay to demonstrate anti-leptospiral activity of IgM and IgG in serum. The titre of IgM detected by the present authors' ELISA method was also higher than that of IgG during the first weeks of the experimental period (three months) in dogs infected with leptospires and also subsequently became virtually equal to the titre of IgG. The titre of IgG did not exceed that of IgM in any of the dogs during the experimental period. This could be due to the difference in affinity of IgM and IgG for antigen. Determining and comparing IgM/IgG ratios is only acceptable when all reagents used in the ELISA test are calibrated. When the

IgM-ELISA was used, a positive response was detected at an earlier stage than it was when the MAT was performed. The superiority of the IgM ELISA is probably due, among other reasons, to the use of a variety of leptospiral antigens prepared by SDS-treatment of leptospires, as a result of which not only agglutinating antibodies are detected. Agglutination obviously requires a larger amount of IgM than does a (positive) response in the ELISA. Determination of the titre of specific anti-leptospiral IgM and IgG in the sera of dogs clinically suspected of leptospirosis by means of the ELISA technique may supply information on the stage of the disease. The present studies showed that high IgM titres will be detectable during the acute stage following infection even when the MAT titre is still negative, whereas the IgG titre will be negative or proportionally very low (Table I). During a subsequent stage, when the MAT titre is stable or decreasing, the differences between the titres of specific anti-leptospiral IgM and IgG will have disappeared (IgM/IgG ratio approximately = 1).

In three out of five dogs the IgM, produced during the initial stage, showed some similarity with rheumatoid factor in that both reacted with IgG. As non-specificity of IgM occurred only during the initial stage after infection with leptospires and did not exist before the experimental infection, it can be assumed that the production of this non-specific IgM was the result of the leptospiral infection. Therefore the titre of the non-specific reaction was not subtracted from the anti-leptospiral IgM titre. However, in the case of a clinically suspected leptospiral infection, the titre of a possible non-specific reaction should be compared with the titre to the leptospiral antigens. If an individual suffers from a current leptospiral infection, the difference between the specific and the non-specific titre should be considerable, as is shown in this paper. The above mentioned false-positive IgM titres could be avoided by coating the specific anti-IgM serum to the solid-phase as described by Yolken and Leister (1981).

The substrate used in the present experiment (5-amino-2-hydroxybenzoic acid) and its enzymic degradation product are partially soluble and therefore only acceptable for visual reading. However, visual reading can be made quantitative by testing a series of dilutions of the test sample (Voller et al., 1978).

At present the soluble substrate ortho-phenylenediamine (OPD) is used in our laboratory and the test is read on a Dynatech Microelisa Minireader MR 590 (wavelength 490 nm). The titre is expressed as the reciprocal of the highest dilution of serum giving an optical density of at least 0.2 (Pope et al., 1982). Comparison of the ELISA titres of sera using both 5-amino-2-hydroxybenzoic acid and OPD showed equal titres with either substrate. The advantage of using OPD as substrate and reading the test spectrophotometrically is an optimal standardization and reproducibility.

CONCLUSIONS

The results of the present study show that the ELISA is a highly sensitive and specific serological technique for the quantitative determination of anti-leptospiral IgM and IgG in canine serum. The test may supply information concerning the stage of the disease and may be of diagnostic importance, particularly during the acute stage of leptospirosis.

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REFERENCES

Adler, B. and Faine, S., 1978. Serological and protective-antibody responses of rabbits to leptospiral antigens. J.Med.Microbiol. 11: 401-409.

Adler, B., Murphy, A.M., Locarnini, S.A. and Faine, S., 1980. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J.Clin.Microbiol. 11: 452-457.

Babudieri, B., 1961. Laboratory diagnosis of leptospirosis. Bull.Wld.Hlth.Org., 24: 45-58.

Bey, R.F. and Johnson, R.C., 1978a. Humoral immune response of dogs vaccinated with leptospiral pentavalent outer envelope and whole culture vaccines. Am.J.Vet.Res., 39: 831-836.

Bey, R.F. and Johnson, R.C., 1978b. Humoral immune response of cattle vaccinated with leptospiral pentavalent outer envelope and whole culture vaccines. Am.J.Vet.Res., 39: 1109-1114.

Crawford, R.P., 1972. Molecular characteristics of antibody detected by the microscopic agglutination test in serum of guinea-pigs with leptospirosis. Am.J.Vet.Res., 33: 1507-1512.

Fathalla, N.C. and Coghlan, J.D., 1980. Detection of leptospiral antibodies in animal sera by means of fractionated antigenic extracts. J.Med.Microbiol., 13: 513-526.

Goudswaard, J., van der Donk, J.A., Noordzij, A., van Dam, R.H. and Vaerman, J.P., 1978. Protein A reactivity of various mammalian immunoglobulins. Scand.J.Immunol., 8: 21-28.

Graves, S.R. and Faine, S., 1970. Antileptospiral agglutinins produced in rabbits. Bull.Wld.Hlth.Org., 43: 579-587.

Hartman, E.G., van Houten, M., van der Donk, J.A. and Frik, J.F., 1984. Serodiagnosis of canine leptospirosis by solid-phase enzyme-linked immunosorbent assay. Vet.Immunol.Immunopathol. (in press).

Heddle, R.J. and Rowley, D., 1975. Dog immunoglobulins. II. The antibacterial properties of dog IgA, IgM and IgG antibodies to *Vibrio cholerae*. Immunology, 29: 197-208.

Hood, L.E., Weissman, I.L. and Wood, W.B., 1978. Immunology. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California.

Kortbeek-Jacobs, N. and van Houten, M., 1982. Oral immunization of sows: anti-K88 antibodies in serum and milk of the sow and in serum of the piglets. The Veterinary Quarterly, 4: 19-22.

Morris, J.A. and Hussaini, S.N., 1974. Characterization of the antibodies detected by the microscopic agglutination test for bovine leptospirosis. J.Hyg.Camb., 73: 425-432.

Negi, S.K., Myers, W.L. and Segre, D., 1971a. Antibody response of cattle to Leptospira pomona. A hemagglutination test to measure IgG and IgM antibodies. Am.J.Vet.Res., 32: 1907-1913.
Negi, S.K., Myers, W.L. and Segre, D., 1971b. Antibody response to cattle to Leptospira pomona: response as measured by hemagglutination, microscopic agglutination and hamster protection tests. Am.J.Vet.Res., 32: 1915-1920.
Pike, R.M., 1967. Antibody heterogeneity and serological reactions. Bacteriol. Rev., 31: 157-174.
Pope, V., Hunter, E.F. and Feeley, J.C., 1982. Evaluation of the microenzyme-linked immunosorbent assay with Treponema pallidum antigen. J.Clin.Microbiol., 15: 630-634.
Sulzer, C.R., Glosser, J.W., Rogers, F., Jones, W.L. and Frix, M., 1975. Evaluation of an indirect hemagglutination test for the diagnosis of human leptospirosis. J.Clin.Microbiol., 2: 218-221.
Tripathy, D.N., Smith, A.R., Hanson, L.E., 1975. Immunoglobulins in cattle vaccinated with letospiral bacterins. Am.J.Vet.Res., 36: 1735-1736.
Turner, L.H., 1968. Leptospirosis II. Trans.R.Soc.trop.Med.Hyg., 62: 880-899.
Voller, A., Bartlett, A. and Bidwell, D.E., 1978. Enzyme immunoassays with special reference to ELISA techniques. J.Clin.Path., 31: 507-520.
Wolff, J.W., 1954. The laboratory diagnosis of leptospirosis. Charles C. Thomas Publ., Springfield, Illinois.
Yolken, R.H. and Leister, F.J., 1981. Enzyme immunoassays for measurement of cytomegalovirus immunoglobulin M antibody. J.Clin.Microbiol., 14: 427-432.