ANTI-RABIES VIRUS IgM IN SERUM AND CEREBROSPINAL FLUID FROM RABID DOGS

MARKPOL TINGPALAPONG, CHARLES H. HOKE, JR., GEORGE S. WARD, DONALD S. BURKE, MICHAEL R. ELWELL, SAKOL LOHYTYOTHIN* and SUPRANEE SAISOMBAT**

Armed Forces Research Institute of Medical Sciences, APO SF 96346. *Thai Red Cross Society Bangkok and, **Department of Livestock Development, Bangkok, Thailand.

INTRODUCTION

Rabies is endemic in Thailand. In a recent report of cases over a 10 year period, between 237 and 322 human cases and between 871 and 3286 canine cases were reported each year (Singhaseni, 1979). With so many annual canine cases and human exposures it is important to have an accurate, reliable method for early diagnosis of canine rabies. At present, dogs which have bitten people are, if caught, quarantined and observed for abnormal behaviour. If a rabies-like illness develops, animals are sacrificed and their brains examined for rabies antigen by an indirect fluorescent antibody (IFA) test.

No serological test exists of value in diagnosing rabies in these dogs. An immunoassay for detection of anti-rabies IgG (Nicholson and Prestage, 1982; Budzko *et al.*, 1983) and an immunoadherence hemagglutination test (Savy, 1978) have been applied successfully to detect antibody in immunized humans. However, IgG antibody is long-lived and its detection is less useful in diagnosis of acute illness than detection of virus specific IgM. Another immunoassay for detection of anti-rabies IgG and IgM, in which antigen is bound to polystyrene plates, demonstrated the appearance of IgG and IgM antibody in humans shortly before death (Atanasiu *et al.*, 1977).

Recently, an IgM antibody capture technique has been developed for detection of virus specific IgM antibody in CSF during human Japanese encephalitis virus infections of the brain (Burke *et al.*, 1982). This test allows rapid diagnosis of most human cases of Japanese encephalitis at the time of admission to the hospital. We wondered whether such a test would be useful in diagnosis of canine rabies. Therefore, we developed an IgM antibody capture radioimmunoassay and evaluated its potential usefulness in diagnosing canine rabies. The purposes of this study were to test this antibody capture radioimmunoassay for anti-rabies IgM (MAC RIA) in serum and CSF of dogs quarantined for rabies observation and to determine the kinetics of IgM antibody appearance in dogs experimentally infected with rabies virus.

MATERIALS AND METHODS

Study animals: Thirty-seven dogs, quarantined for rabies observation at the Thai Red Cross Society in Bangkok, Thailand were entered in this study. For CSF sampling dogs were anesthetized with 0.4 mg/kg intramuscular xyladrol (Bay Vet, Div. of Cutter, Shawnee, Kansas). Blood from the jugular vein and cerebrospinal fluid from the *cysterna magna* were obtained. Generally, dogs had bitten people and were quarantined because

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of abnormal behavior, so samples were generally taken from already ill dogs.

Rabies confirmation: When a dog died, 0.03 ml of a 20% suspension of its brain in phosphate buffered saline, pH 7.2, and 0.03 ml CSF were each injected intracerebrally into 6 mice. The 12 mice were observed for thirty days. If mice died, impression smears of their brain were examined by IFA. Dogs were classified as rabid if they died with an illness clinically compatible with rabies (abnormal behavior, excessive salivation, jaw paralysis and abnormal attempts to bite followed by preterminal generalized paralysis) and had positive intracerebral mouse inoculation tests. If dogs remained healthy they were classified as non-rabid. Dogs dying of an illness compatible with canine distemper (intermittent convulsion, chronic mucopurulent discharge from eye and nose, abdominal pustules, and terminal exhaustion) and free of laboratory evidence of rabies virus were classified as non-rabid.

Experimental rabies: The rabies virus used for inoculation had been isolated from a dog confirmed to be rabid by the IFA test and passed one time in mice before using. CSF and serum from experimented mongrel dogs were tested 4 times for baseline rabies MAC RIA values over a 2 month period preceding infection. Two dogs were given 100 mice LD_{50} of rabies virus titered in weanling mice in the neck muscles; 2 dogs were given a commercial modified live virus vaccine (Merieux Institute, Lyon, France) and one dog, a control saline injection. The latter 3 injections were made in the thigh muscle. Serum and CSF were sampled twice weekly until death or 45 days post-infection.

Rabies MAC RIA: Specimens were tested without knowledge of the clinical history of the dogs. A commercially prepared rabbit anti-dog IgM (Miles Labs, Inc., P.O. Box 2000, Elkhart, Indiana) was used without modification. It was diluted 1:200 in carbonate buffer at pH 9.5. and 100 microliters was placed in each test well of 96 well polystyrene microtiter plates and incubated overnight at 4°C. The plates were washed 3 times in phosphate buffered saline containing 0.05% Tween 20 (PBS-T), and test specimens were applied. Sera were diluted 1:100 in 20% acetone extracted normal rabbit serum and CSF specimens were diluted 1:10 in the same diluent. Fifty microliters of diluted specimens were placed in each test well of the plates and incubated for 4 hours in a humidified box at room temperature. The plates were washed 3 times in PBS-T. Merieux rabies vaccine (Merieux Institute, Lyon, France) was diluted 1:16 in 20% acetone extracted normal rabbit serum and 50 microliters of the diluted antigen were placed in test wells and incubated overnight at 4°C. Several other preparations of rabies antigen were tried, but none worked better than vaccine antigen. The plates were washed 3 more times. Rabbit anti-rabies gamma-globulin, which had been purified from hyperimmunized rabbit antisera (kindly supplied by Drs. K.G. Nicholson and Mary Warrell) by ammonium sulfate precipitation and chromatography, was conjugated with ^{125}I using the chloramine-T method (Greenwood and Hunter, 1963) performed on the day the tagged antibody was used. The iodinated anti-rabies antibody was diluted in 20% acetone extracted normal rabbit serum to approximately 150,000 counts per minute. Twenty-five microliters of the tagged antibody were placed in each test well and incubated at room temperature for two hours. After washing 7 times, the polystyrene plates were cut into individual wells. The remaining radioactivity in each well was determined by counting disintegrations for one minute in a Packard gamma counter. To confirm that IgM antibody was being assayed, sera were fractionated on 5-20% sucrose gradients and each fraction was tested.

Rheumatoid Factor: Rheumatoid factor was assayed using the differential sheep cell test (Rose, *et al.*, 1948). The courtesy of the Division of Immunology, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital.

Analysis: Data were analyzed by comparing the mean cpm (counts per minute) of the CSF and serum of the two groups of dogs tested. Paired or un-paired t-tests were used as appropriate.

RESULTS

During a 2 month period all 37 dogs brought to the quarantine facility during the working day were included in the study (Table 1). 24 dogs died within one to five days of a rabies-like illness, and rabies virus infection was confirmed by mouse inoculation in 21. CSF from 6 of the 21 dogs also contained infectious rabies virus. Three dogs died with clinical signs of rabies but the brains and CSFs of these animals were negative for rabies by mouse inoculation, so they were classified as possible rabies.. Twelve dogs remained well, were released, and are assumed to have been non-rabid. One dog died of apparent clinical canine distemper after 2 days of quarantine. Its brain contained no rabies virus, and it is included as a non-rabid dog.

Rabies MAC RIA test: To demonstrate that IgM was being detected in the test, sucrose density gradient fractionation of CSF was performed. Substantially elevated counts were detected in fraction 3 of CSF from a rabid dog, the usual location of IgM.

Tests for rheumatoid factor on all specimens with elevated counts in the rabies MAC RIA test were negative, indicating that rheumatoid factor did not account for binding in the rabies MAC RIA.

Titration of serum and CSF from dogs from three groups were performed. Serum from two rabid dogs had titers of 1:1000, and CSF, 1:100. Consequently it was elected to test further serum specimens at 1:100 dilution and CSF at 1:10 dilutions. Specimens from a dog having a rabies-like illness but a negative IFA gave higher counts at each dilution than specimens from dogs with positive IFA tests.

CPM in serum and CSF from rabid dogs were significantly higher than in specimens from non-rabid dogs ($P \leq .001$ for both) (Fig.1). CSF and serum from normal dogs

Table 1

Clinical and laboratory data for rabies in dogs studied.

	Total	Rabies-like illness No.	Brain IFA *	Brain*	CSF *
Normal dogs	12	0	NT	NT	0
Rabid group	21	21	21	21	6
Distemper group	1	0	0	0	0
Possible rabies	3	3	0	0	0
Total	37	24	21	21	6

NT = Not tested.

* Number of dogs positive, mouse inoculation test.

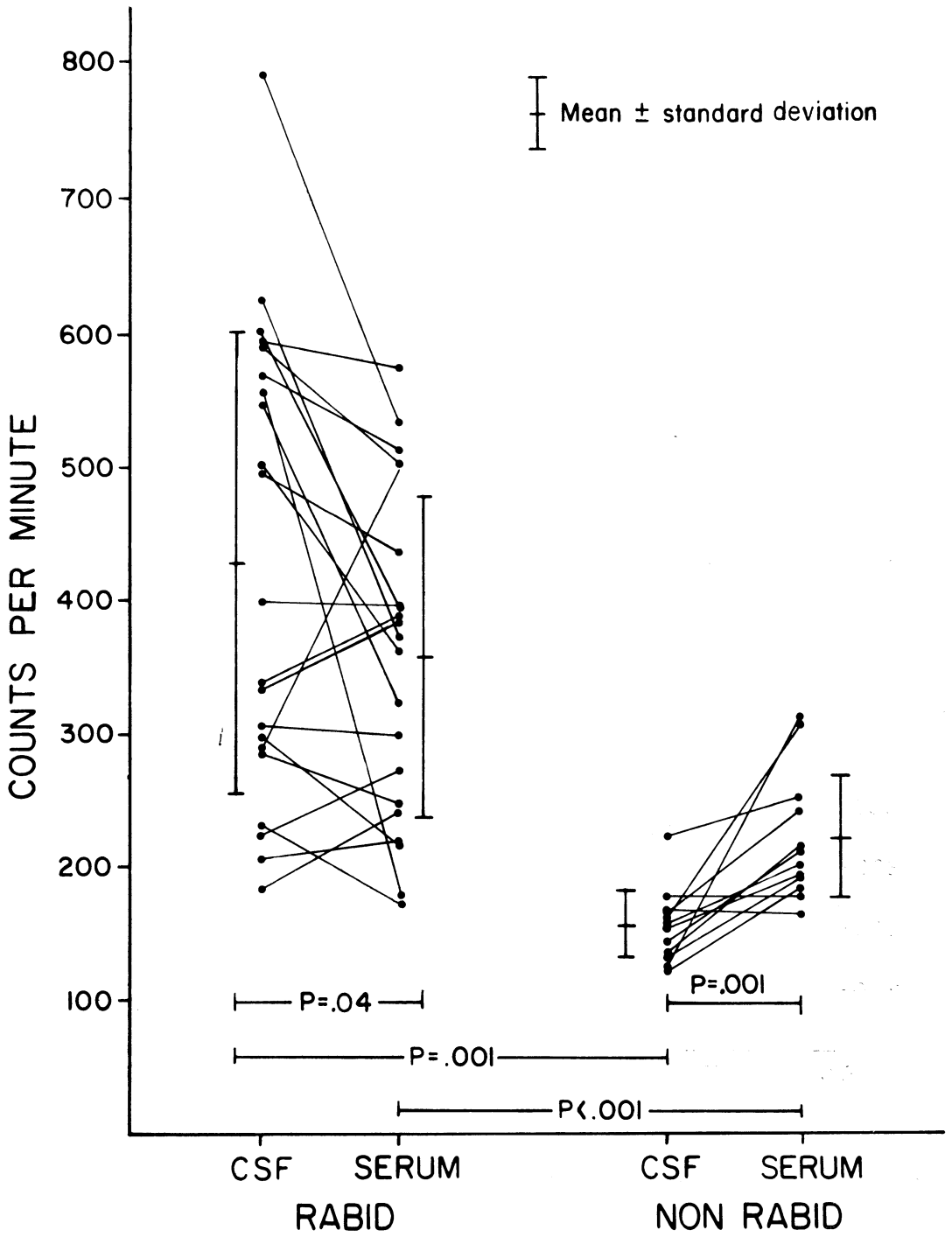

Fig. 1—Rabies MAC RIA results in CSF and serum of rabid and non-rabid quarantined dogs.

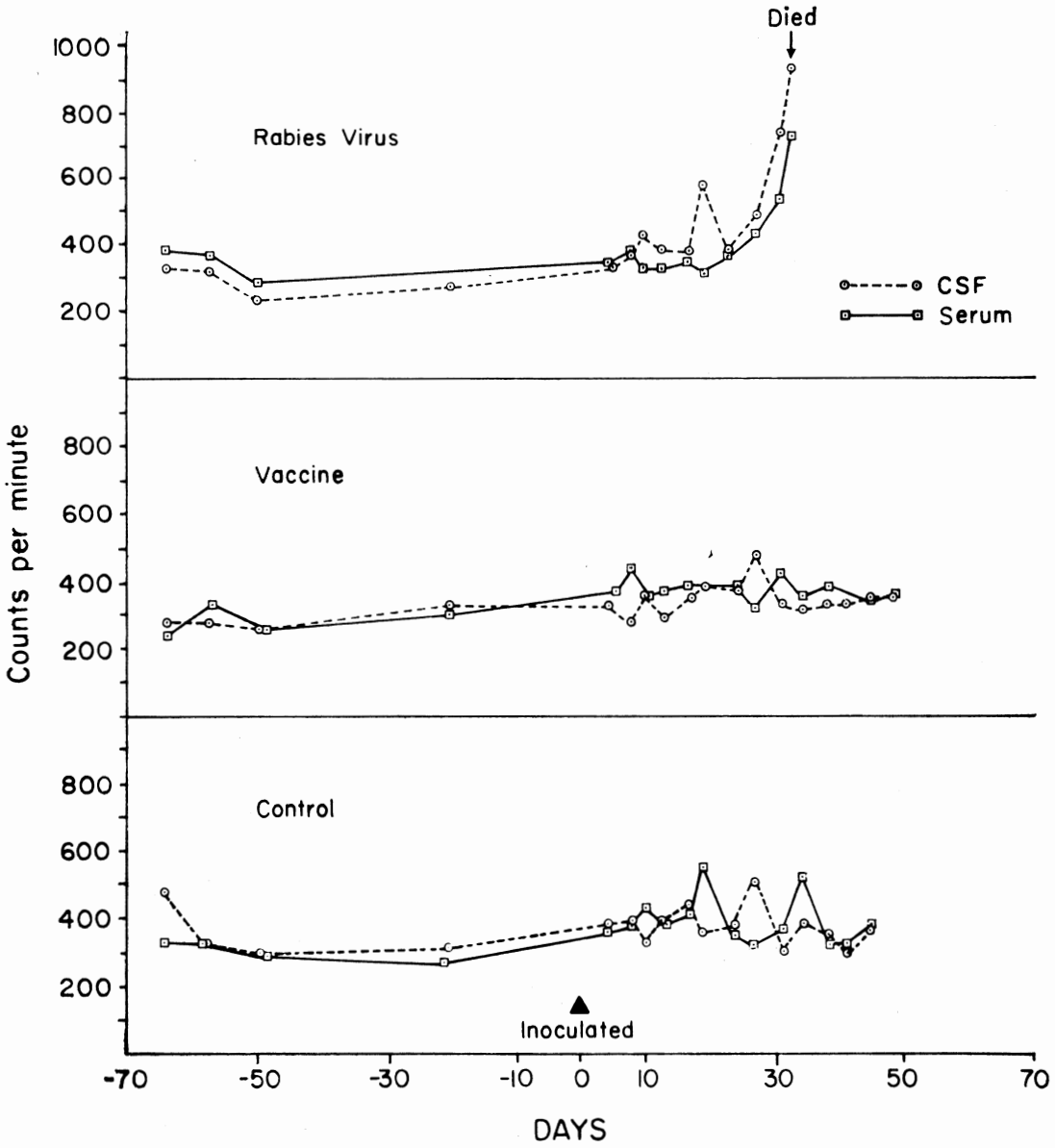

Fig. 2—Anti-rabies IgM antibody capture RIA result in a rabid dog, an immunized dog, and a control dog.

gave significantly lower CPM in CSF than serum. By contrast, from rabid dogs, CPM in CSF were significantly higher than in serum ($p<.04$). In some dogs, CSF counts were elevated several fold over serum counts, suggesting that antibody may on occasion be synthesized in the CSF.

To determine the possible clinical utility of the test, two different cut off points for positive and negative values were compared. Optimum results were obtained when CSF was tested and a cut off of the mean plus 2 standard deviations of the negatives was used (sensitivity=90%, specificity =92%, predictive value of a positive test = 95%) (Table 2,) while other cut off points were markedly less sensitive.

Experimental rabies: Rabies virus was inoculated into two dogs. One failed to become ill. One became ill 31 days after inoculation and died on day 34 (Fig. 2). Anti rabies IgM appeared in CSF and serum at about the same time as clinical illness developed, and rose until the time of death 3 days later. Thus, the development of antibody seemed related in time to progression of the disease. IFA and mouse inoculation test were positive at the time of death.

In dogs which had been given one dose of rabies vaccine and in control dogs, no elevation of IgM was detected.

DISCUSSION

We had hoped that an IgM antibody test, analogous to that successfully employed to diagnose Japanese encephalitis in humans would be a useful tool for diagnosis of rabies encephalitis in dogs. Sucrose density fractionation demonstrated that the test detected a substance with the molecular weight of IgM. The absence of rheumatoid factor in any specimen tested demonstrates that these results are not due to such a substance.

Titrations revealed serum titers of about 1:1000 and CSF titers of 1:100, both of which are rather low compared with human Japanese encephalitis (Burke *et al.*, 1982). Single dilutions for further testing (1:10 for CSF and 1:100 for serum) were chosen on the basis of these results.

CSF and serum from rabid dogs gave significantly elevated counts as compared to similar specimens from non-rabid dogs, demonstrating that the disease could be diagnosed using this test (Fig. 1). The range of CPM's in CSF from non-rabid dogs (122-223) over-lapped the range of CSF from rabid dogs

Table 2

Evaluation of two cut of criteria for diagnosing rabies using MAC RIA.

Cut off	Specimen	Correctly classified Rabies (N=21)	Non (N=13)	Sensitivity	Specificity	Predictive Value
$\bar{x}$ + 2SD (212)	CSF	19	12	.90	.92	.95
x × 2.1 (330)	CSF	13	13	.60	1.00	1.00
$\bar{x}$ + 2SD (313)	SER	13	13	.61	1.00	1.00
$\bar{x}$ × 2.1 (454)	SER	5	13	.23	1.00	1.00

(182-791) only slightly, while serum ranges over-lapped considerably more (165-312 in normal dogs vs 172-528 in rabid dogs), suggesting that tests on CSF, rather than serum, would best discriminate rabid from non-rabid animals.

Moreover, CSF counts were substantially elevated over serum counts in several dogs, a result which was never obtained in well dogs. While some blood brain barrier penetration of antibody may have occurred, synthesis within CSF is also possible. Evidence for such synthesis of antibody within CSF also exists for human Japanese encephalitis (Burke *et al.*, 1982).

Since most quarantined animals were ill when sampled, it was necessary to learn from an experimental animal when anti-rabies IgM antibody appeared during the clinical course. An animal with experimental rabies was followed through the full course of his illness (Fig. 2). No evidence of anti-rabies IgM appeared until approximately 3 days before death, just before the appearance of clinical symptoms. At that time, IgM CPM's rose to well above base levels, consistent with results for humans (Atanasiu *et al.*, 1977). More data is needed to understand the relationship between clinical illness and the appearance of antibody. In rabies encephalitis, illness seems to progress once antibody appears. While it is possible that clinical illness and antibody develop as the result of a common causal factor such as virus replication, it is also possible that the antibody contributes to the pathogenesis of the disease.

Particularly interesting were two of the 3 quarantined dogs, dying with clinical evidence of rabies. In these two dogs, unusually high counts were obtained (983 and 685 counts in CSF, 825 and 382 in CSF) despite the absence of IFA detectable or culturable virus in the brain tissue. Three possible explanations exist for these findings: (1) The dogs were rabid but our IFA test and culture system failed to detect virus; (2) the dogs were not rabid and our tests detected IgM antibody against some other etiologic agent; (3) the dogs were rabid, but the IFA test and culture were negative because no free antigen or virus was present as the result of high levels of blocking antibody.

We favor the third hypothesis. First, our culture system was sensitive enough to detect rabies virus in CSF in 6 cases, an unusual finding, and the IFA test is routinely done with appropriate positive controls. Second, if the dogs were in fact not rabid, it is unlikely that the MAC RIA test would have been positive, since there are two steps which enhance the specificity of the test: Our rabies antigen was a highly purified rabies vaccine and anti-rabies antibody which we purified and tagged was obtained from a rabbit hyperimmunized with rabies virus.

The third possibility would occur if anti-rabies antibody blocked antigen, preventing detection in the IFA test and neutralizing virus, preventing cultivation in the mouse inoculation test. Two of these dogs had among the highest results of those tested in the rabies MAC RIA test, suggesting that the most antibody was present in them. If substantiated, this result implies that a substantial number of rabies cases in animals may be missed if the IFA test alone is relied upon, leading to under-administration of post-exposure prophylaxis to bitten humans.

In conclusion, we tested an IgM anti rabies-antibody capture radio immunoassay. The assay detects IgM antibody, not rheumatoid factor, at titers of 1:1000 in serum and 1:100 in CSF. The test may be useful for diagnosing or confirming rabies in clinically ill dogs, particularly dogs with high levels of antibody that may block IFA tests. Anti-rabies IgM antibody appears as clinical symptoms develop and rises until the time of death. Further studies to refine the test by reducing background levels, improving sensitivity and con-

verting it to an ELISA for general use are needed. In addition studies to determine the role of the immune system in the pathogenesis of rabies may be facilitated by the use of this test.

SUMMARY

An anti-rabies IgM antibody capture radio immunoassay was used to test serum and cerebrospinal fluid from 37 dogs held in quarantine for suspicion of rabies. Rabies was confirmed in dogs that died by mouse inoculation and subsequent examination of mouse brains by fluorescent antibody technique to detect rabies antigen. The mean counts per minute (CPM) of iodinated anti-rabies gamma globulin coupled IgM rabies antibody in CSF and serum from rabid dogs were significantly higher than in CSF and serum from non-rabid dogs. Mean CPM from rabid dogs was greater in CSF than in sera, in contrast with non-rabid dogs, from which mean cpm was higher in sera than CSF, suggesting that antibody may have been synthesized in the CSF. To evaluate this test further, a dog was infected by rabies virus, and serial serum and CSF specimens were collected until the time of death. IgM anti-rabies antibody developed in the CSF and serum 29 days following infection, and rose just before the dog died of rabies on day 34.

The rabies MAC RIA is potentially useful as a diagnostic method in quarantined dogs with rabies-like illness. Perhaps more importantly, it may be applied to better understand the immunopathogenicity of rabies.

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