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Competitive Radioimmunoassay To Detect Antibodies to Herpes B Virus and SA8 Virus

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A monoclonal competitive radioimmunoassay (CompRIAm) which detects antibody to herpesvirus simiae (B virus) in monkey and human sera and antibody to SA8 virus in monkey sera but not antibody to herpes simplex virus in human sera is described. Of 232 serum samples from wild-caught cynomolgus monkeys, 117 serum samples were positive when tested by CompRIAm. The results were in close agreement (97.5%) with B virus neutralizing antibody results on the same sera. Sera from 97 wild-caught rhesus monkeys and 92 wild-caught baboons were also tested. The CompRIAm was able to differentiate between sera that had neutralizing antibody to B virus and SA8 virus and those that did not, although the discrimination was not as clear as that in the tests on cynomolgus monkey sera. Sequential sera from two humans with confirmed cases of B virus infection were tested by CompRIAm. B virus antibody was detected in sera from both humans. None of 237 other serum samples from blood donors and patients attending sexually transmitted disease clinics reacted in the CompRIAm.

Herpesvirus simiae (B virus) is an alpha herpesvirus of macaque monkeys. It is very closely related to herpes simplex virus (HSV) of humans. B virus infection in macaques is generally subclinical and resembles human HSV infection. There is a primary oral or genital infection followed by virus latency in the nerve ganglia. In humans, however, B virus can cause a devastating neurological illness. Of the 25 well-documented human B virus infections, an ascending encephalomyelitis was diagnosed in 22 patients, and in 16 of these (72%) patients the outcome was fatal (3, 6, 13). Simian agent 8 (SA8) is a closely related alpha herpesvirus of baboons and African green monkeys. It has not been shown unequivocally to infect humans, but it may have been the cause of one fatal case of encephalitis (10). The apparent success of early acyclovir therapy in controlling clinical illness caused by B virus in humans emphasizes the need for prompt and accurate laboratory diagnosis (6).

B virus and HSV share growth characteristics and antigens, and it is difficult to distinguish by culture or serologically between B virus infection and HSV infection. Primary diagnosis of B virus infection is by isolation of the virus in cell culture. B virus identification has been described by indirect immunofluorescence with monoclonal antibodies (MAbs) (2) or by restriction enzyme digestion of viral DNA (12). Isolation of virus is not always possible, however, and it is important to be able to diagnose B virus infection serologically. Several methods for detecting B virus antibody have been described: neutralization (7), a dot immunoassay (5), and an enzyme-linked immunosorbent assay (ELISA) (8). Only the ELISA, which is based on cross-absorption of antibodies, appears to discriminate reliably between B virus and HSV antibodies. We describe here the development of a monoclonal competitive radioimmunoassay (CompRIAm) which detects antibody responses to B virus and SA8 but not to HSVs.

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MATERIALS AND METHODS

Viruses. B virus strain Cyno 2 is an oral isolate from a cynomolgus monkey (*Macaca fascicularis*) (11), and strain Sabin was obtained from the American Type Culture Collection (ATCC VR-126). The 1401 strain of SA8 used was obtained from J. Hilliard, Southwest Foundation for Biochemical Research, San Antonio, Tex., and the SC16 strain of HSV type 1 was obtained from the North Regional Virus Laboratory, Manchester, United Kingdom.

Sera. The simian sera were collected from 232 wild-caught cynomolgus monkeys from Indonesia and the Philippines, 92 wild-caught Ethiopian baboons, and 97 wild-caught Chinese rhesus monkeys. These sera were submitted to the Virus Reference Division for antibody testing during 1990.

Several groups of human sera were examined: 189 serum samples were from blood donors in north London in 1990 and 48 serum samples were from patients attending a sexually transmitted diseases clinic in south London. A total of 12 human serum samples known to be positive for rheumatoid factor were also examined; these were provided by Wendy Knowles (Virus Reference Division). A total of 24 pairs of human serum samples showing rises in titer to either HSV or varicella-zoster virus (VZV) were provided by Hilar Kangro (Department of Virology, St. Bartholomews' Hospital, London, United Kingdom) and M. Hambling (Public Health Laboratory, Leeds, United Kingdom). Human sera and cerebrospinal fluid (CSF) from two patients with human B virus infection (patients 1 and 2) (1, 4) were tested. These were kindly provided by R. Fmmons, Viral and Rickettsial Diseases Laboratory, Department of Health Services, Berkeley, Calif.

Antigen preparation. Vero cells were grown in 80-cm^2 flasks in Eagle's modified minimal essential medium with Earle's balanced salt solution supplemented with 5% fetal calf serum. When confluent, the medium was aspirated and the cells were washed once in phosphate-buffered saline (PBS). The cells were then infected with virus at a multiplicity of infection of 0.5 PFU per cell. After 1 h of adsorption, the inoculum was removed and the cells were washed once in PBS and then 20 ml of serum-free Eagle's modified

minimal essential medium with Hanks salt solution was added to the cells. When a 4+ cytopathic effect was observed (usually after 24 h), the flasks were freeze-thawed three times and sonicated (with a probe sonicator on ice). Formaldehyde was added to give a 5% final concentration, and the mixture was left at room temperature for 120 min with constant agitation. The preparation was then divided into aliquots, and they were stored at −70°C until required. Mock-infected cells were produced by a similar method.

MAbs. The B virus-specific MAbs were prepared as described previously (2). The suitability of the MAbs for use in a solid-phase diagnostic assay was assessed. Antigen-coated beads were incubated overnight at 37°C with monoclonal hybridoma fluids diluted at 1:100 in PBS with 0.05% Tween 20 and 5% fetal calf serum. The beads were then washed twice in PBS with 0.05% Tween 20. Bound MAb was detected by using ^{125}I-labeled anti-mouse antibody (Amersham International, Aylesbury, Buckinghamshire, United Kingdom; IM131). The MAbs were tested in this assay format against B virus Cyno 2 strain, B virus Sabin strain, SA8, HSV, and mock antigen. The ratio of counts bound to test antigen/counts bound to mock antigen was calculated for each virus. MAb C30.1 was selected for use in the assay since it gave a binding ratio of 52.2 against the Cyno 2 strain of B virus and a binding ratio of <1 against all the other viruses tested. This MAb has been characterized previously and was shown to neutralize B virus (2).

Competitive assay. Etched polystyrene beads (diameter, 6.5 mm; Northumbria Biologicals Ltd.) were coated with the antigen by rolling the beads overnight at 37°C in antigen diluted 1:25 in 0.1 M carbonate-bicarbonate buffer (pH 9.6; Don Whitley Scientific Ltd.). The optimal dilution for coating was previously determined by cross-titration of antigen dilutions against MAb dilutions. After coating, the optimally diluted antigen was aspirated and replaced with PBS containing 1% bovine serum albumin and 0.05% Tween 20 and the mixture was incubated for 1 h at 37°C to quench unbound sites.

Sera were tested in recycled 20-well plates originally supplied with Abbott immunoassay kits. Test serum (20 μl) was pipetted into an empty well of the plate; this was followed by the addition of 200 μl of a 1/1,000 dilution of MAb. An antigen-coated bead was added to each well, and the mixture was incubated overnight at 37°C. The beads were then washed twice in PBS containing 0.05% Tween 20. A total of 200 μl of ^{125}I-labeled sheep anti-mouse antibody (Amersham, IM131) diluted in PBS–Tween 20 with 10% fetal calf serum to give approximately 80,000 counts per minute in 200 μl was added to each bead, and the mixture was incubated at 37°C for 6 h. The beads were then washed twice in PBS containing 0.05% Tween 20 before being transferred to tubes and counted in a gamma counter for 60 s.

Results were quantified by measuring the percent reduction in binding of MAb 30.1 as follows: % inhibition of MAb = $100\times [1 - (c_t/c_n)]$, where c_n is the count given by a reference negative serum sample, and c_t is the count given by the test serum sample.

Neutralization assay. The neutralizing antibody titers of serum specimens against B virus (strain Cyno 2) and SA8 (strain 1401) were determined by incubating serial dilutions of them with a constant dose (100 50% tissue culture infective doses) of each virus as described previously (9).

Polyclonal competitive radioimmunoassay. A polyclonal competitive radioimmunoassay was used. The assay was previously shown to detect antibody to HSV, B virus, and SA8 but not VZV (data not shown). Briefly, polystyrene balls were coated with B virus antigen which was previously detergent solubilized and inactivated by exposure to 3×10^4 Gy by using a ^{60}Co source. The beads were incubated with a mixture of test serum and mouse ascitic fluid raised against B virus. Binding of mouse antibodies was detected by incubation with a ^{125}iodine-labeled anti-mouse antibody (Amersham, IM131). The results were expressed as percent inhibition of binding of mouse antibodies by the test serum compared with that of a B virus antibody-negative monkey serum pool. Test sera giving >60% inhibition were regarded as positive.

RESULTS

Reactivity of cynomolgus monkey sera in the CompRIAm. A total of 232 cynomolgus monkey serum samples were tested in the CompRIAm. The distribution of reactivities of these serum samples is shown in Fig. 1. The sera tested fell into two distinct groups, indicating good discrimination by the assay. The results were used to set a cutoff level for the assay. In previous tests for neutralizing antibody, 123 of the serum samples were shown to contain antibodies against B virus; 117 (95%) of them gave >45% inhibition by CompRIAm. The mean inhibition given by these 117 serum samples was 83.5% (standard deviation, ±9.25%). On the basis of these results, the positive cutoff value for CompRIAm was set at 56% inhibition (3 standard deviations below the mean). The remaining 109 serum samples, which were negative by neutralization, gave inhibitions in the CompRIAm of less than 35%. Their mean inhibition was 8.8% (standard deviation, ±6.9%). On this basis, a negative cutoff was set at 30% inhibition. Test sera giving inhibitions of between 30 and 56% were considered equivocal. By using these criteria, there was 95% agreement between CompRIAm and B virus neutralization. The six serum samples that gave discrepant results were collected from wild-caught cynomolgus monkeys from the Philippines and Indonesia. Five of these six serum samples had low serum neutralization titers (titer, 4).

Reactivity of human sera in the CompRIAm. A total of 237 human serum samples were tested by CompRIAm. These sera were previously tested by polyclonal competitive radioimmunoassay, and 116 (49%) serum samples were shown to contain herpesvirus antibodies. None of the human sera tested reacted positively in the CompRIAm.

Reactivities of paired human sera showing rises in titer to VZV or HSV in the CompRIAm. Some 24 pairs of serum samples were tested in the CompRIAm. Of these, 10 showed rises in titer to VZV, 2 showed rises in titer to HSV, and 12 showed rises in titer to both VZV and HSV when tested in the complement fixation test. None of these acute-phase or convalescent-phase sera gave positive results when tested by CompRIAm.

Reactivities of sera and CSF from two humans with B virus infection in the CompRIAm. Sequential serum samples and a CSF sample from patient 1 were tested. He had an encephalomyelitic illness caused by B virus; he recovered from the illness (1). Serum from another patient (patient 2) was tested; the patient recovered from the illness (4). The results are presented in Table 1. Significant reactivity was detected in the CompRIAm in sera collected from both patients and in the CSF of patient 1.

Reactivities of rhesus monkey sera in the CompRIAm. A total of 97 rhesus monkey serum samples were tested by CompRIAm. The distribution of reactivities of these sera is shown in Fig. 2. The sera fell into two distinct groups,

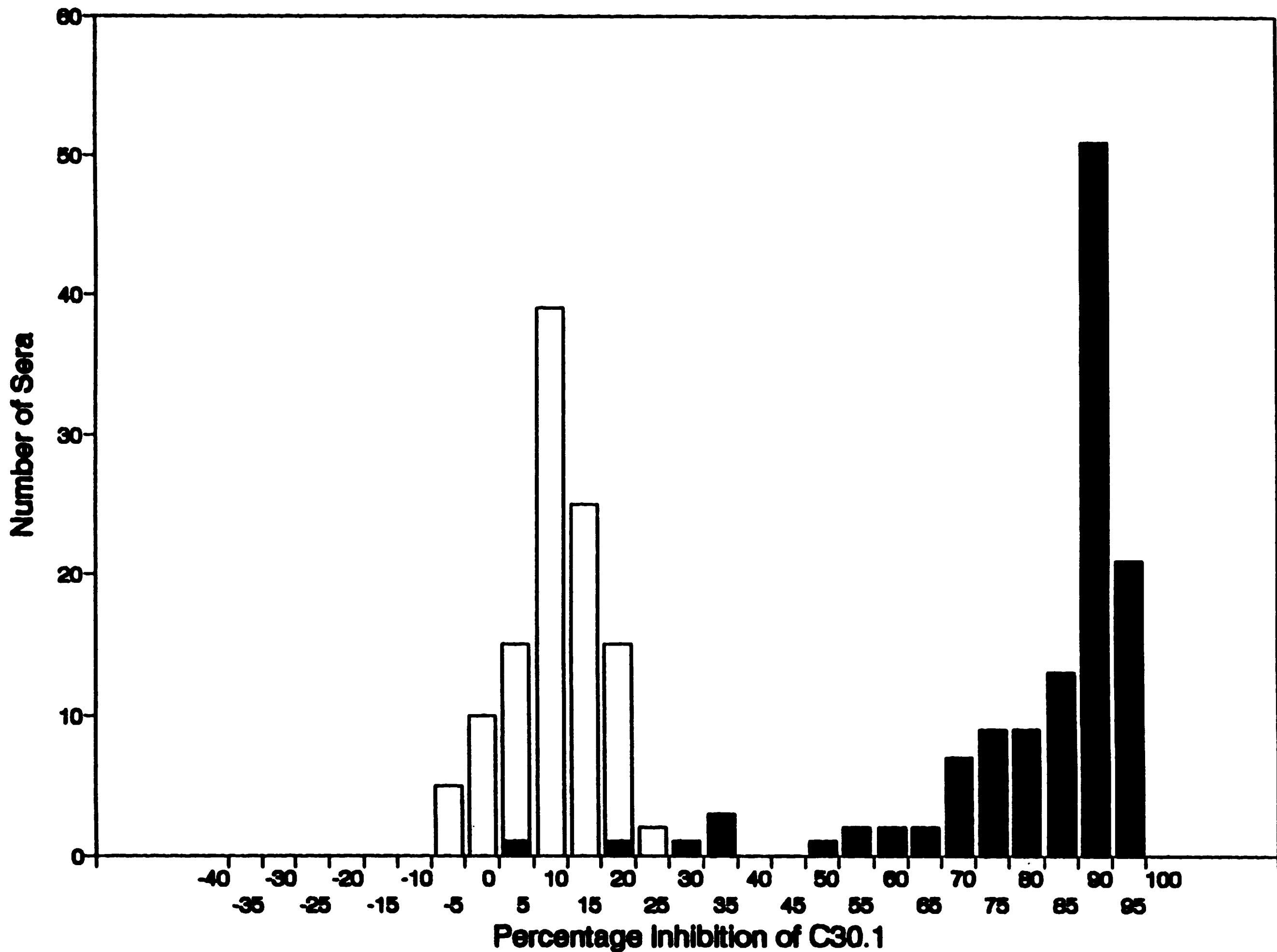

FIG. 1. Percent inhibitions of 232 cynomolgus monkey serum samples tested in the CompRIAm. ■, sera previously tested by neutralization and shown to contain antibodies against B virus; □, sera negative for antibodies against B virus when tested by the neutralization assay.

indicating discrimination between B virus antibody-positive and -negative sera. There was 85% agreement between neutralization and CompRIAm on rhesus monkey sera.

Reactivities of baboon sera in the CompRIAm. A total of 92 baboon serum samples were tested by CompRIAm. The sera fell into two distinct groups, indicating that the assay could discriminate between seropositive and seronegative animals. There was 84% agreement between neutralization and CompRIAm on baboon sera.

TABLE 1. Reactivities of sera and CSF samples collected from two human B virus cases in polyclonal and monoclonal antibody-based CompRIA

Patient	Specimen	Time after onset (days)	% Inhibition (group)[a]	
			Polyclonal competitive RIA	Monoclonal CompRIAm
1	Serum	8	89.2 (+)	33.0 (equivocal)
	Serum	22	86.3 (+)	45.3 (equivocal)
	Serum	117	91.9 (+)	91.0 (+)
	CSF		82.8 (+)	59.6 (+)
2	Serum	37	91.8 (+)	68.4 (+)
	Serum	814	90.9 (+)	31.8 (equivocal)

[a] Positive result for inhibition of binding, >56%.

Statistical analysis of the positive distributions of the five populations tested. The distributions of percent inhibitions given by the five groups of sera tested (cynomolgus monkey, rhesus monkey, baboon, human blood donors, and human sexually transmitted disease clinic attendees) were examined statistically by a nonparametric test to look at differences in their medians, and these were shown to be different ($P < 0.001$). Inspection of the 95% confidence intervals of the medians suggested that the samples fell into three groups with distinct medians: those for human blood donors and human sexually transmitted disease clinic attendees were low, those for rhesus and baboon monkeys were intermediate, and those for cynomolgus monkeys were high.

DISCUSSION

Specific serological diagnosis of B virus infection has proved difficult, particularly in suspected human B virus cases, because of the many antigens shared between HSV

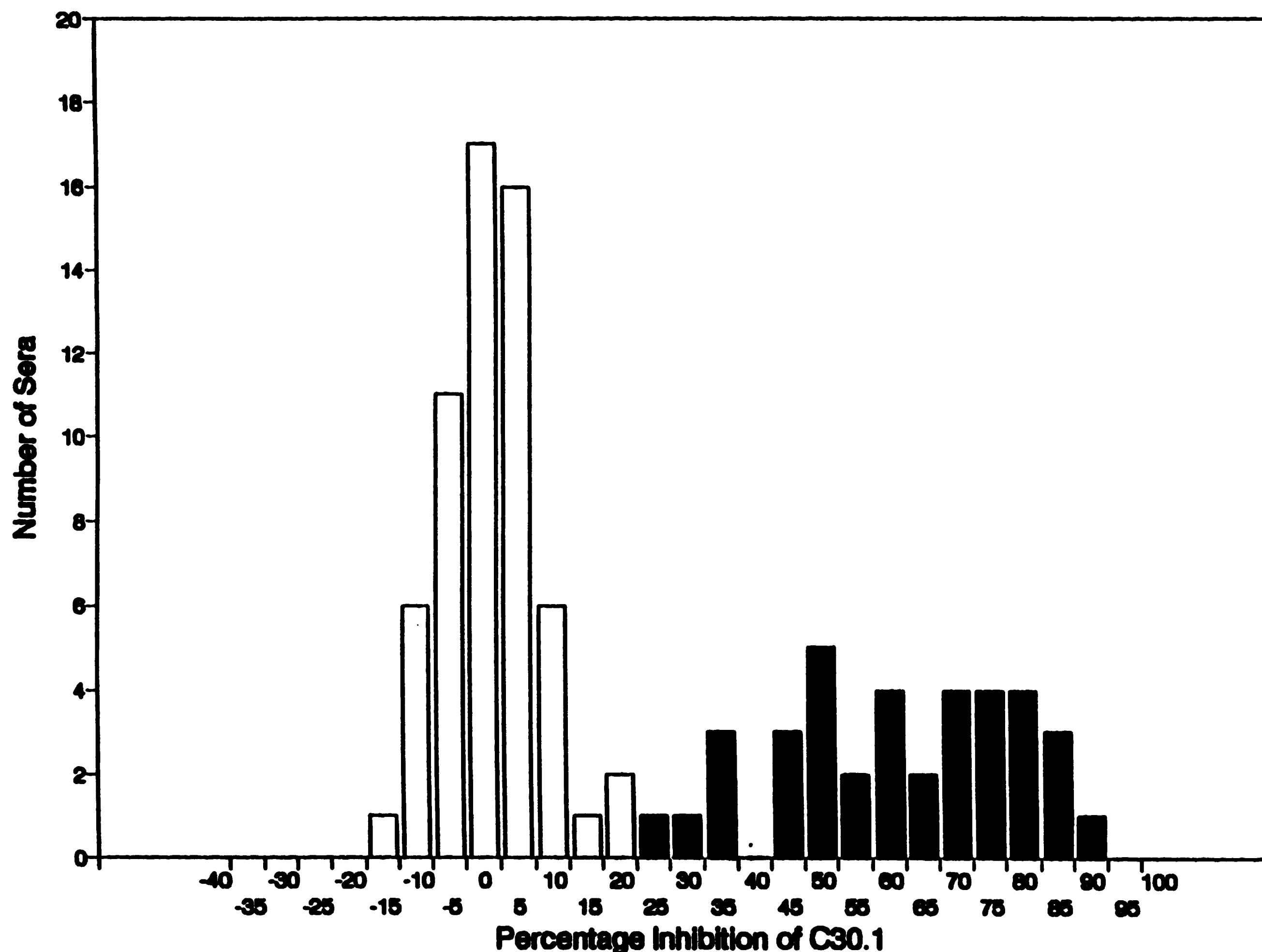

FIG. 2. Percent inhibition of 97 rhesus monkey serum samples tested in the CompRIAm. ■, sera previously tested by neutralization assay and shown to contain antibodies against B virus; □, sera negative for antibodies against B virus when tested by the neutralization assay.

and B virus. An ELISA which reliably detects B virus antibodies, even in the presence of HSV antibodies, has been described, but it is based on laborious preabsorption of cross-reactive antibodies (8). The CompRIAm described here is simpler to perform and uses inactivated antigen, thus avoiding the need to perform laboratory work with this hazardous organism in a safety cabinet.

The MAb C30.1 used in the assay reacted only against the strains of B virus from cynomolgus monkeys in the solid-phase immunoassay. Despite this, sera from both baboon and rhesus monkeys inhibited MAb C30.1 binding to the Cyno 2 antigen preparation. The antibodies detected in these rhesus and baboon sera are likely to be directed against their indigenous strains, B virus and SA8, respectively, since all sera were collected from newly captured wild animals. The average percent inhibition in CompRIAm detected in seroreactive baboon and rhesus sera was significantly lower than that in sera from cynomolgus monkeys. The basis for this difference is unknown, since we have been unable to define the protein target of the MAb by Western blot (immunoblot) or immunoprecipitation, but it may represent stearic hindrance by antibody binding to an adjacent site or the effect of lower-avidity antibody to the Cyno 2 epitope generated in response to the related viruses of baboon and rhesus monkeys. It is fortunate for diagnosis of B virus infections in humans that the assay detects antibodies directed against rhesus B virus strains efficiently, since human B virus infections have been linked almost exclusively with exposure to rhesus monkeys.

None of the human sera screened gave a positive result in the assay; even sera containing high-titer antibody responses following recent HSV or VZV infection failed to react. This supports the specificity of the CompRIAm. The sensitivity of CompRIAm for detecting past B virus infection in cynomolgus monkeys showed good agreement with that of the serum neutralization assay. The explanation for the few discrepant results is unclear, but the neutralization-positive, CompRIAm-negative sera may have contained antibody to a related but different herpesvirus, such as a B virus strain lacking the relevant Cyno 2 epitope, or they may have been of human origin since, in some facilities, newly captured monkeys are given injections of human gamma globulin. Alternatively, this may represent a lack of sensitivity of the CompRIAm.

We were able to detect B virus antibodies by CompRIAm in sera collected from two patients reported to have B virus

infection and in the CSF of one patient. The rate of development of human B virus immune responses could not be fully assessed in the present study, but it appears to be delayed since, in patient 1, B virus antibody developed between 22 and 117 days after the onset of clinical illness. In patient 2, B virus antibody was present on day 37. The detection of B virus antibody against a background of preexisting HSV antibody illustrates the potential value of the assay in the diagnosis of acute human B virus infection. Studies are under way to establish whether it can be used to examine sera from workers who are in regular contact with monkeys to look for the possibility of B virus latency in humans.

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