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Heterophil Antigen in Bovine Sera Detectable by Immune Adherence Hemagglutination with Infectious Mononucleosis Sera

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Bovine sera for tissue culture use were shown to contain heterophil antigen of the Paul-Bunnell-Davidsohn type by immune adherence hemagglutination tests with sera from patients with infectious mononucleosis. The antibody titers observed were comparable to those determined by two other methods; i.e., the immune adherence hemagglutination test was found to be as specific as the differential absorption test with horse erythrocytes or the ox cell hemolysis assay, but it appeared to be the most sensitive of the three procedures. All 16 individual or pooled bovine serum samples showed Paul-Bunnell-Davidsohn antigen at concentrations varying over a fourfold range. The potential usefulness of the immune adherence hemagglutination test for various Paul-Bunnell-Davidsohn antigen-related problems and implications of the observations are discussed.

In the immune adherence hemagglutination (IAHA) assay, antigen-antibody-complement complexes are bound to C3-b receptors on primate erythrocytes (RBC), leading to agglutination of the cells (14). The procedure has proved sensitive for the detection of specific antigens and has been applied successfully to titrations of antibodies to varicella-zoster virus, cytomegalovirus, herpes simplex virus, hepatitis virus types A and B, and a number of enteroviruses (2, 3, 8, 11, 13). The IAHA titers of antigens or antibodies are 4- to 10-fold higher than those obtained by human complement fixation tests and are comparable to those determined by immunofluorescence.

In attempts to adapt this method to the detection of antibodies to the Epstein-Barr virus (EBV), the cause of infectious mononucleosis (IM) (4), spent media from virus-producing lymphoblastoid cell lines were tried as sources of viral antigen. Surprisingly, only a majority of sera from patients with acute IM gave positive IAHA reactions, but none of the sera from healthy donors did, despite the fact that they had substantial titers of antibodies to EB viral capsid antigen. Furthermore, spent media from nonproducer lymphoblastoid cell lines, fresh culture media, and even fetal bovine serum (FBS), the serum component of the culture media, also reacted with the IM sera in the IAHA test. These observations suggested that bovine sera contain heterophil antigen of the Paul-Bunnell-Davidsohn (PBD) type (1). The present report describes the usefulness of the IAHA test for the detection of PBD antigen and the corresponding antibodies.

MATERIALS AND METHODS

Human sera. Human sera were selected from a collection of frozen specimens that had been submitted for EBV-specific serodiagnostic tests. They were divided into four categories: (i) current primary EBV infections, (ii) EBV infections in the past, (iii) no previous EBV infections, and (iv) IM-like illnesses due to cytomegalovirus. The techniques used for the various EBV-specific serological procedures have been described (5). The essential criteria for differentiation of groups i, ii, and iii are shown in Table 1. All sera in group iv gave evidence of EBV infections in the past. The majority of sera in group i but not in group iv contained IM-specific heterophil antibodies, as determined by horse RBC agglutination in the differential absorption test described previously (6, 9). For the IAHA test, the sera were diluted 1:5 in Veronal-buffered saline (VBS) solution and inactivated at 56°C for 30 min.

Reagents for the IAHA test. The reagents for the IAHA test were: (i) 0.05 M phosphate-buffered saline solution containing (per liter) 70 ml of 0.2 M NaH_2PO_4, 180 ml of 0.2 M Na_2HPO, and 1.63 g of NaCl; (ii) 5× stock of VBS containing (per liter) 41.9 g of NaCl, 1.26 g of $NaHCO_3$, 1.5 g of sodium 5,5-diethylbarbiturate, 2.3 g of 5,5-diethylbarbituric acid, 0.5 g of $MgCl_2 \cdot 6H_2O$, and 0.2 g of $CaCl_2$ (isotonic VBS was prepared from the stock by fivefold dilution with deionized water);

TABLE 1. *Groups of sera selected for study*

Donors	Antibodies to:				
	VCA[a]		D[d]	EBNA[e]	PBD
	IgM[b]	IgG[c]			
Current IM	+	+	+/0	0	+/0
Past EBV infection	0	+	0	+	0
No EBV infection	0	0	0	0	0

[a] VCA, Viral capsid antigen.
[b] IgM, Immunoglobulin M.
[c] IgG, Immunoglobulin G.
[d] D, Diffuse component of the early antigens.
[e] EBNA, EBV-associated nuclear antigen.

(iii) gelatin VBS, prepared by adding 0.125% autoclaved gelatin to VBS; (iv) bovine serum albumin VBS, prepared by adding 1 mg of bovine serum albumin fraction V per ml of VBS; and (v) dithiothreitol-ethylenediaminetetraacetate-VBS, containing 2 parts of 0.10 M ethylenediaminetetraacetate, 3 parts of VBS, and 3 mg of dithiothreitol per ml (Sigma Chemical Co.).

Human type O RBC. About one in three donors was found to provide type O RBC suitable for the IAHA test. Whole blood was collected under aseptic conditions in 2 volumes of Alsever solution and used after storage at 4°C for at most 5 weeks. The RBC were washed twice in VBS, washed once in gelatin VBS at 5°C, and finally suspended to a concentration of 0.75% in gelatin VBS.

Guinea pig complement. Commercially obtained guinea pig complement (Cordis Laboratories) was diluted in bovine serum albumin VBS just before use. A dilution of 1:90 to 1:100 proved optimal.

Heterophil antigen. Pooled fetal bovine serum for tissue culture (Microbiological Associates, Inc.) served as the standard source for heterophil antigen. The serum was usually diluted 1:10 in 0.05 M phosphate-buffered saline for the IAHA test. Individual newborn calf sera and bovine amniotic fluids were kindly furnished by R. Spendlove, Sterile Systems Inc.

Microtiter plates. Many of the microtiter plates from different sources were found unsuitable for the IAHA test. Both nontissue and tissue culture grade V plates from mold no. 14 (Lindbro Scientific Inc.) proved satisfactory. Shortly before use, the plates were rinsed with gelatin VBS, inverted, and rapped to drain off excess fluid. This precoating step eliminated most nonspecific reactions.

IAHA test. Except for a few modifications, the procedures were those described by Gershon et al. (3). Each reagent was added in 1 drop of 0.025 ml. Bovine serum albumin VBS was placed in each well of the precoated microtiter plate. A drop of test serum was then added to the first wells of two rows, and serial twofold dilutions were prepared. Heterophil antigen (FBS) in 0.05 M phosphate-buffered saline was added to one of the rows, and phosphate-buffered saline was added to the other. The plates were vibrated and incubated at 37°C for 30 min. After addition of complement, the plates were shaken and returned to 37°C for 40 min. Dithiothreitol-ethylenediaminetetraacetate-VBS was then added to stop the reaction and was followed immediately by the addition of the human type O RBC suspension. The plates were kept at room temperature, and the hemagglutination patterns were readable 1 to 2 h later. Agglutination of 3+ or greater was considered positive.

Serum absorptions. Serum absorptions were carried out in microtiter plates. The fivefold-diluted test sera were mixed with equal volumes of a 10% suspension of bovine RBC or a guinea pig kidney suspension (Difco). After incubation at 37°C for 20 min, the plates were shaken and centrifuged at 1,000 × *g* for 15 min. The supernatants were removed and used for IAHA and ox cell hemolysis tests.

Ox cell hemolysis assay. The procedure for the ox cell hemolysis assay described by Mikkelsen et al. (12) was adapted for microtiter plates. The sera were serially diluted in VBS as described for the IAHA test. After the addition to each well of 1 drop of 0.025 ml of a 1% suspension of twice-washed bovine RBC in VBS and 1 drop of complement diluted 1:15, the plates were incubated at 37°C for 0.5 h and read for the degree of hemolysis. Sets of serum dilutions without complement served as controls for nonspecific hemolysis. A serum titer of 1:40 or greater was considered positive.

RESULTS

Titration of PBD antibodies by IAHA. Sera from 42 donors without antibodies to EBV, 57 healthy individuals after past primary EBV infections, 74 patients within 30 days of the onset of IM, and 51 patients with cytomegalovirus-related illnesses were titrated against FBS in IAHA tests. The results (Fig. 1) showed that 97 of the 99 (98%) donors with no or past EBV infections had titers of <1:40. In contrast, 67 of

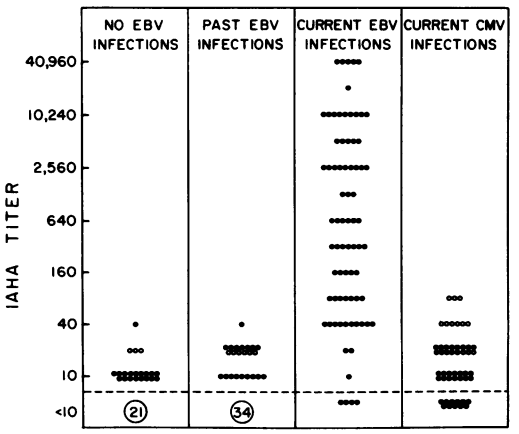

FIG. 1. *IAHA antibody titers obtained with FBS antigen and sera from individuals without EBV infection (42), past EBV infection (57), current primary EBV infection (74), and cytomegalovirus-induced mononucleosis-like illnesses (51). Symbols: ●, specific IAHA reactions; ○, serum control showing nonspecific hemagglutination.*

the 74 (91%) acute IM patients gave titers of ≧1:40, with a median of 1:1,280. These results indicated that the reactive component in FBS is heterophil antigen of the PBD type. With a titer of 1:40 as the dividing line betwen positive and negative, as in the ox cell hemolysis test, only 9% of the IM patients gave negative results, and only 2% of the two control groups yielded barely positive results. Of the 51 patients with cytomegalovirus-induced IM-like illnesses, 9 had titers of 1:40 or 1:80, but the serum controls, tested against phosphate-buffered saline instead of FBS, gave similar results, which thus were nonspecific.

Comparisons between the antibody titers measured by the tests of IAHA, ox cell hemolysis, and horse RBC agglutination (after guinea pig kidney absorption) are presented in Fig. 2. The sera from 96 patients with current or recent IM chosen for these comparisons had shown virus-related antibody patterns characteristic of current primary EBV infections (Table 1) and horse RBC agglutinin titers ranging from <1:7 to ≧100,000. There were excellent correlations between antibody titers ≧1:40 as determined by the IAHA and the horse RBC agglutination tests and between results of the IAHA and the ox cell hemolysis tests; there was a slightly less close correlation between the results of the horse RBC hemagglutination and hemolysis assays. Among sera showing horse RBC hemagglutinin titers of <7, the IAHA test still picked up a few with titers of 1:40 or 1:80, which were considered positive. This was further substantiated by the analysis of 74 sera collected within 30 days after onset of IM (Table 2), which showed that 21 (28%) were negative in the ox cell hemolysis test, 13 (18%) were negative in the horse RBC agglutination assay, and 7 (9%) were negative in the IAHA procedure. The seven IAHA negative sera were also negative in the other two tests.

To confirm that the antibodies detected by the IAHA test were directed against PBD antigen, 19 sera from IM patients with IAHA titers of ≧1:40 were adsorbed with bovine erythrocytes or guinea pig kidney suspension. The bovine RBC reduced the titers to <1:20 (Fig. 3). Absorption of the sera with guinea pig kidney suspension failed to decrease the IAHA titers (not shown).

PBD antigen in different bovine sera. To determine whether the presence of PBD antigen in the lot of FBS used in the above experiments was fortuitous or not, seven commercial lots of pooled FBS for tissue culture use from two suppliers, nine sera (SS-1 to SS-9) from individual newborn calves, and eight individual am-

TABLE 2. *Comparison of overall results obtained by IAHA, horse RBC agglutination, and ox cell hemolysis tests*[a]

Assay	IAHA	
	Positive	Negative
IAHA		
Positive	67	0
Negative	0	7
Horse RBC agglutination		
Positive	61	0
Negative	6	7
Ox cell hemolysis		
Positive	53	0
Negative	14	7

[a] Positive serum titers: IAHA and ox cell hemolysis, ≧1:40; horse RBC agglutination, ≧1:7.

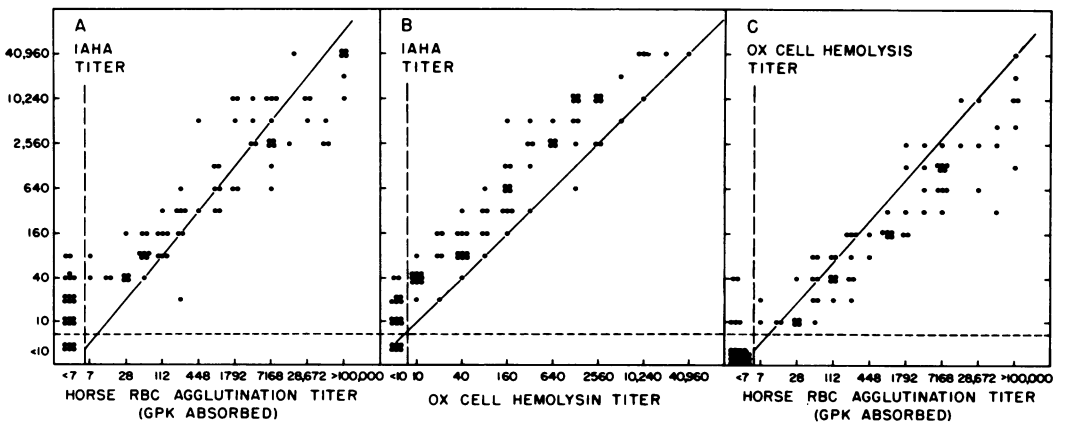

FIG. 2. *Relation between heterophil antibody titers of 96 sera from IM patients. Shown are comparisons of the results obtained by (A) the IAHA and differential horse RBC agglutination tests, (B) the IAHA and ox cell hemolysis tests, and (C) the differential horse RBC agglutination and ox cell hemolysis tests. The diagonal lines indicate the slope of equal titers. GPK, Guinea pig kidney suspension.*

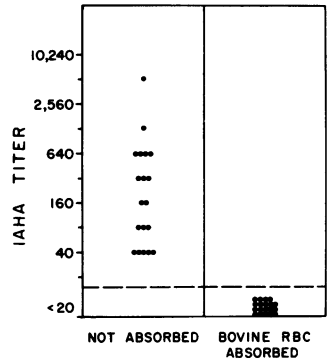

FIG. 3. *Heterophil antibody titers obtained in the IAHA test with 19 sera from IM patients before and after absorption with bovine RBC. Absorption with guinea pig kidney suspension did not reduce the titers.*

niotic fluids were tested in checkerboard titrations. Serial twofold dilutions of bovine sera and amniotic fluids (1:2 to 1:32) were tested against serial twofold dilutions of a standard, heterophil antibody-positive serum from an IM patient. The optimal antigen dilutions of the FBS samples varied between 1:8 and 1:32 (Table 3). In contrast to the relatively narrow range of antigen activity, the maximal titers of the reference IM serum ranged from 1:160 to 1:10,240. It is noteworthy that pooled FBS from one supplier gave uniform results with respect to optimal antigen dilution and the antibody titers of the test serum. The variations were noted with individual bovine sera and a FBS pool from another source. The bovine amniotic fluids contained at most small, presumably suboptimal, amounts of PBD antigen, so that the reference serum titers were of a low order.

TABLE 3. *Optimal PBD antigen titers in bovine sera*

Bovine specimen	Optimal antigen titer	Reference IM serum titer
Pooled serum		
MBA 82411	1:8	1:10,240
MBA 88386	1:8	1:10,240
MBA 84199	1:8	1:10,240
MBA 91474	1:8	1:10,240
MBA 91373	1:16	1:10,240
MBA 40551058	1:8	1:10,240
Hyclone 00119	1:16	1:640
Individual serum		
SS-1	1:16	1:2,560
SS-2	1:8	1:160
SS-3	1:8	1:160
SS-4	1:16	1:2,560
SS-5	1:16	1:2,560
SS-6	1:16	1:5,120
SS-7	1:16	1:2,560
SS-8	1:32	1:1,280
SS-9	1:8	1:5,120
Individual amniotic fluid		
1	≤1:2	1:40
2	≤1:2	1:40
3	≤1:2	1:80
4	≤1:2	1:40
5	≤1:2	<1:40
6	≤1:2	1:80
7	1:4	1:80
8	1:4	1:160

DISCUSSION

The above results indicate that bovine sera contain heterophil antigen of the PBD type and that the IAHA procedure is useful and sensitive for the detection of soluble PBD antigen and the corresponding antibodies. For measuring IM-specific heterophil antibodies, the IAHA test is no less specific and possibly somewhat more sensitive than the differential absorption test performed with horse RBC. Compared with the hemagglutination procedure, the IAHA test has the further advantage that no absorption of the test sera is required. This advantage is shared by the ox cell hemolysis assay, which, however, is distinctly less sensitive than the IAHA test. Thus, the IAHA test is a potentially useful alternative to the other two assays. As the IAHA technique may become applicable in time to the detection and titration of antibodies to EBV-related antigens and might then replace some of the presently employed immunofluorescence tests, the addition of pooled FBS as antigen for parallel titration of PBD antibodies would be a simple procedure. A small number of sera gave nonspecific IAHA reactions in the absence of antigen, perhaps because of the presence of antigen-antibody complexes in the sera.

The discovery of PBD type heterophil antigen in bovine serum was unexpected. However, agglutination of horse serum-coated latex particles by IM sera has been reported (10). All bovine sera tested thus far revealed PBD antigen, with optimal doses ranging from 1:8 to 1:32. It is currently unexplained why the maximal antibody titers of a standard IM serum should vary so greatly (from 1:160 to 1:10,240) with the use of different bovine sera as antigen. Several suggestions deserve further exploration. (i) There may be several distinct PBD components that are represented to different extents in bovine sera and to which the sera of IM patients may have antibodies at different titers. Differences in the collection and processing of bovine sera could result in varying degrees of hemolysis or release of antigenic components from the RBC

(if the RBC are the source of the antigen in FBS) and could account for the variable reactivities of individual bovine sera, which would be averaged in large pools. No correlation between color and clarity of bovine sera and their reactivity in the IAHA test has so far been noted. (ii) The PBD antigen might be present in the form of aggregates or breakdown products, which again could depend on the methods of collection and processing of the sera. The size of the antigenic components could conceivably affect the antibody titers obtained by the IAHA test. (iii) Factors inhibiting or enhancing immune adherence might be present in some bovine sera. Characterization of the PBD antigen(s) in different bovine sera is required to distinguish between these possible explanations.

As the IAHA test led to the detection of PBD antigen in FBS, it should be a useful assay in the search for this antigen in other fluids or in tissues. PBD antigen has not been found in significant amounts in samples of bovine amniotic fluid. Up to now, soluble PBD antigen could be detected in a specific manner only by its blocking effect in hemagglutination or hemolysis tests, which often yield equivocal results because of their limited sensitivity. The presence of PBD antigen in FBS in soluble form should permit its purification and characterization. Preliminary observations indicate that the antigen(s) can be separated from bovine serum and partially purified with relative ease and that the IAHA test greatly facilitates tracking of the antigen during fractionation.

Several recent reports have shown that bovine serum components are incorporated into the membrane of cultured cells maintained in media containing FBS and that the acquired antigens may influence the results of lymphocytotoxicity assays (7, 15). The bovine serum component(s) involved has (have) not been identified but may well include PBD antigen. This possibility must be considered in studies of immune responses to target cells grown in the presence of FBS, whether cell mediated, humoral, or a combination of both.

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