Cold Agglutinins in Infectious Mononucleosis and Heterophil-Antibody-negative Mononucleosis-like Syndromes

By Charles A. Horwitz, John Moulds, Werner Henle, Gertrude Henle, Herbert Polesky, Henry H. Balfour, Jr., Burton Schwartz, and Tom Hoff

Cold agglutinins (CA) were evaluated prospectively in patients with various mononucleosis syndromes and in a large control group. Cold agglutinins with anti-i specificity were seen mainly in heterophil-positive or -negative Epstein-Barr virus (EBV)-induced infectious mononucleosis (31.8% of cases). Unclassified CA with equal reactivity against cord and adult erythrocytes were seen in 56 of 150 (37.3%) cases of heterophil-antibody–positive infectious mononucleosis (IM), in 1 of 7 (14.3%) cases of heterophil-negative EBV-induced IM, and in 12 of 31 (38.7%) cases of the heterophil-negative mononucleosis-like syndrome due to cytomegalovirus or other unspecified agents. One patient with heterophil-positive IM had a persistent, partially papain sensitive CA with anti-Pr–like activity. Anti-i CA were seen in <1.0% of healthy young adults (500) or patients without mononucleosis (500) submitted for heterophil studies. Unclassified CA were noted in 3.2% of the latter 1000 samples.

COLD AUTOANTIBODIES reacting preferentially with cord (i) red blood cells rather than adult (I) cells (i.e., anti-i) are seldom seen in normal control populations,[1,2] occasionally seen in lymphoproliferative disorders and other disease states,[3,4] and often encountered in patients with heterophil-antibody–positive Epstein-Barr virus (EBV)-induced infectious mononucleosis (IM).[5-9] Their incidence during the acute phase of IM has been reported to occur in from 8% to 60%–70% of patients. This variability has been attributed to the methods used for detection as well as the patient population under study.[10] Cold agglutinins (CA) also appear during the course of the heterophil-antibody–negative mononucleosis syndrome due to cytomegalovirus (CMV).

Previous studies of CA in the latter syndrome have reported only tests using adult red blood cells (i.e., the detection of anti-I).[11-14] This paper reviews a prospective study of CA in patients with IM, including 38 cases of the heterophil-antibody–negative mononucleosis-like syndrome. The antibody specificities observed are compared to results from tests on 1250 control samples.

From the Departments of Laboratory Medicine and Internal Medicine, Mount Sinai Hospital, War Memorial Blood Bank and the University of Minnesota Medical School, Minneapolis, Minn.; the Division of Virology, Joseph Stokes Jr. Research Institute, the Children's Hospital of Pennsylvania, and the School of Medicine, University of Pennsylvania, Philadelphia, Pa.

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Address for reprint requests: Charles A. Horwitz, M.D., Department of Laboratory Medicine, Mount Sinai Hospital, 2215 Park Avenue, Minneapolis, Minn. 55404.

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MATERIALS AND METHODS

Standard Differential Cold Agglutinin (CA) Studies

The test sera were diluted in duplicate in twofold steps with normal saline in 13 × 100 mm glass test tubes starting at 1:7. Thrice-washed 2%-3% suspensions of type O human adult erythrocytes were added to one of the rows of serum dilutions, and cord erythrocytes were added to the other. Cord cells supplied on a weekly basis from the War Memorial Blood Bank, Minneapolis, Minn., were tested against a panel of known anti-i sera before utilization in this study. Adult cell suspensions were made from routine samples submitted to the blood bank of Mount Sinai Hospital. A strong anti-i control serum was included in all CA experiments. The serum and red blood cell suspensions were well mixed and then incubated overnight at 4°-6°C. Endpoints against cord and adult erythrocytes were read simultaneously. The ×10 lens of a standard light microscope was used to confirm marginal macroscopic agglutination. When agglutination was observed after 4°C incubation, samples were tested for disagglutination by further incubation at room temperature (22°C) or in some cases in a 37°C waterbath. Those CA that showed complete or significant disagglutination were then classified into the following four categories depending on the relative titer against cord or adult erythrocytes: (1) anti-i, when the CA titer with cord red blood cells was two or more tubes greater than the simultaneously performed titer against adult cells (when the CA titer was clearly positive at 1:7 against cord cells but negative (<1:7) against adult red blood cells, the patient was also considered to have anti-i); (2) unclassified, when CA titers were of equal strength with cord and adult erythrocytes or when there was only a one-tube difference between the titers; (3) anti-I, when the CA titer with adult erythrocytes was at least two tubes greater than the simultaneously performed titer against cord erythrocytes; or (4) negative results, when no CA were observed or when the reaction with adult erythrocytes was limited to the 1:7 dilution and the cord erythrocytes did not agglutinate (<1:7).

Detection of 7S Low Molecular Weight CA

For the detection of IgG anti-i equal amounts of serum and 0.1 *M* 2-mercaptoethanol (2-ME) were incubated for 1 hr at 37°C and then tested (4°C) by an indirect antiglobulin test with monospecific rabbit anti-human gamma-G globulin. Selected sera also were tested by this procedure following incubation with dithiothreitol (DTT).

Epstein-Barr Virus-related Serology

Antibodies to EBV-related antigens were determined by immunofluorescence techniques. Antibodies to viral capsid antigens (VCA) were titrated by a routine procedure using EB-3 cells of Burkitt lymphoma origin (producer line).[15] Antibodies to the early antigen (EA) were determined with EBV superinfected Raji cells that are normally free of VCA or EA but carry the EBV genomes (nonproducer line).[16,17] The Raji lines were superinfected with concentrated suspensions of EBV from a producer line and the cell smears that were prepared 48 hr later fixed in either acetone or methanol. The former were used for detection of antibodies to the diffuse and/or restricted components of the EBV-determined EA, and the latter, for antibodies to only the diffuse component.

Antibodies to the EBV-associated nuclear antigen (EBNA) were determined by an anticomplement immunofluorescence technique using nonsuperinfected Raji cell smears as previously described.[18,19] IgM antibodies to VCA were detected by indirect immunofluorescence using smears of HR-1 cells of Burkitt lymphoma origin which had been maintained at 32°C for 10 days without refeeding and fluorescein-conjugated goat antibodies to human IgM (DaKopatts A/S). CMV complement-fixing (CF) titers were determined by standard complement fixation procedures using an antigen prepared from the AD-169 strain of human fibroblasts as supplied by Microbiological Associates, Bethesda, Md. CMV-macroglobulin (CMV-IgM) titers were determined by immunofluorescence using test kits supplied by Virgo Diagnostics, Bethesda, Md.

Case Material

1. Serial serum specimens were obtained from 38 cases of the heterophil-antibody-negative mononucleosis-like syndrome, of which 29 were due to CMV, 7 to EBV, and 2 to unspecified agents. In all CMV-induced IM cases, anti-CMV-IgM were present in acute specimens and sig-

nificant titers ($\geq$ 1:32) of anti-CMV were detected in serial specimens by the complement fixation technique. These CMV sera showed no evidence of recent EBV activity, i.e., no VCA-IgM antibodies (<1:10) and stable, moderate antibody titers to VCA-IgG and EBNA.

In the 7 cases of heterophil-negative, EBV-induced IM, EBV studies showed IgM-VCA antibodies and the initial absence of anti-EBNA in 6 cases, significant levels of VCA-IgG ($\geq$ 1:320) in 3 cases, and the detection of anti-D of the EA complex in 4 cases. The serial sera clearly indicated ongoing primary EBV infections.[19] Positive results were seen also in the CMV-IgM test on 3 of 7 of the latter cases. This result was due to the known "cross-reaction" of EBV-induced IM sera in the CMV-IgM test.[20] However, acute CMV infection sera do not generally cross-react in the VCA-specific IgM test.[21,22] In the remaining 2 cases, neither EBV nor CMV could be implicated as the cause of the patients' mononucleosis-like syndromes.

2. Serial specimens were obtained from 150 consecutive patients with heterophil-antibody positive IM.

3. Controls included single specimens from 500 healthy young adults, 22 healthy hospital employees, and 500 consecutive patients (excluding groups 1 and 2 above) on whom heterophil studies had been requested by the referring clinician. All of these sera were negative in heterophil studies with horse erythrocytes.

4. Also tested were single specimens from 228 patients with rheumatoid arthritis (53), malignant lymphoma (27), serum paraprotein spikes (19), other conditions (84)—mainly with polyclonal hypergammaglobulinemia and individuals (45) whose serum samples had shown elevated CA in the routine hospital serology laboratory.

RESULTS

The standard differential CA test on *initial* specimens from 150 patients with heterophil-positive IM showed that 30 (20.0%) had anti-i, 62 (41.3%) were unclassified, 33 (22.0%) had anti-I, and 25 (16.7%) had negative CA patterns. On initial specimens from the 38 various heterophil-negative mononucleosis syndromes (29 due to CMV, 7 due to EBV, and 2 due to unspecified agents), 3* (7.9%) had anti-i, 11 (28.9%) unclassified, 15 (39.5%) anti-I, and 9 (23.7%) negative CA patterns. When data from *serial* specimens from both heterophil-positive and the heterophil-negative syndromes (188) were classified on the basis of highest CA titer during the acute illness (Fig. 1), the incidence of anti-i was 20.1% (38 cases), unclassified pattern 43.2% (81), anti-I 25.5% (48), and negative results 11.2% (21). Differential titers at 22° and/or 37°C revealed anti-i in an additional 17.6% (12/68) of heterophil-positive cases originally categorized as having "unclassified" CA at 4°C. With these tests, no additional anti-i was found in patients with mononucleosis-like syndromes due to CMV or other unspecified agents.

Preillness specimens were available from 15 cases of heterophil-positive IM, 12 drawn 45 days or more (up to 4 yr) before the onset of acute illness. Of the 15 specimens, 14 showed no anti-i cell CA activity (<1:7) and were either negative specimens, (11 $\leq$ 1:7) or had 1:14–1:28 (3 specimens) CA titers against adult cells. The 15th specimen showed anti-i cell titers of 1:112 and anti-I of 1:224.

A breakdown of CA patterns seen in single specimens for various control groups is presented in Table 1. Anti-i was found in 0.8% of all control specimens and in only 1 of 500 samples submitted for heterophil studies where accompanying blood smears showed "minimal reactivity."

*All three patients had EBV-induced IM.

CA Titer	Anti-i (Cord Cells)		Unclassified (Cord-Adult Cells)		Anti-I (Adult Cells)	
	Het-Pos	Het-Neg	Het-Pos	Het-Neg	Het-Pos	Het-Neg
3584	•		••	○		
1792	••		•			
896	••••••		••		○	○
448	•••		••••	○○	•	•
224	••••••		••••••			○○
112	•••••	•••	••••••• •••••••		••••••	○○○○○
56	••		••••••• •••••	○○○○○	•••••	○○○○
28	•••		••••••• ••	○‡‡	••••••• •••	○
14	•••		••••••• ••••••• ••	•	•••••••	○○○○
7	••••		••	○	••••••• ••	●○
< 7					••••••• •	●○
Totals	35	3	68	13	47	22
	38 (20.1%)		81 (43.2%)		69 (36.7%)	

Fig. 1. CA in heterophil-positive IM (150 cases) and heterophil-negative IM-like disease (38 cases). Cases were classified by comparison of CA titers (4°C) simultaneously performed against cord (i) and adult (I) erythrocyte suspensions. For this figure "negative" results (10 cases) were included in the anti-I columns; 17.6% (12/68) of "unclassified" (4°C) sera from heterophil-positive IM revealed anti-i specificity with incubation at 22° or 37°C. Het-Pos, heterophil-antibody–positive; Het-Neg, heterophil-antibody–negative; CA, cold agglutinins; IM, infectious mononucleosis. ●, Epstein-Barr virus; ○, cytomegalovirus; ‡, unspecified cause.

Six specimens from the control group with anti-i CA patterns (Table 1) were tested for EBV-related antibodies, and none showed evidence of ongoing active EBV infection. Five of these six specimens also gave negative results (<1:16) for the CMV-IgM. The sixth specimen was from a 29-yr-old woman with seropositive (1:640) rheumatoid arthritis. Such sera with antigammaglobulin factors interfere in some viral IgM tests, including the CMV-IgM test.[23]

Detection of IgG Cold Agglutinins

Of 21 specimens (10 anti-i, 9 unclassified, and 2 anti-I) 20 were negative for 7S CA after treatment with 2-ME. One specimen showed a weakly positive anti-i cell titer (1:7) but was negative (<1:7) against adult cells. This result could not be confirmed on repeat testing several days later. Two samples with known IgG and anti-Rh antibodies were used as controls for the IgG-CA procedure. Five anti-i specimens were negative for 7S CA after treatment with DTT.

Special Studies

Papain treatment. Twenty-four specimens, originally categorized as anti-i (3), anti-I (2), or unclassified (19), were retested for CA activity against both untreated and papainized test cells. Of the 24 specimens, 23 showed higher titers against papainized cells. The other specimen, received 16 days after onset of uncomplicated heterophil-positive IM from a 24-yr-old patient (Frio), failed to show the expected increased titer with papainized cells. Serial sera from this patient were subjected to further studies (see below).

Temperature differential study. The 19 unclassified samples were tested at 16°, 10°, and 4°C in an effort to detect examples of anti-i that may have been masked by preexisting anti-I. Controls included samples with anti-i (3) and anti-I (2). Of 14 heterophil-positive sera, 3 met our criteria for anti-i reactivity

Table 1. Cold Agglutinins in Control Groups

Group No.	Source of Specimens		Anti-i*	Unclassified*	Anti-I* ≧ 1:14)	Negative (≦ 1:7)
I	Healthy student controls (mainly ages 18–23)	500	1	12	99	388
II	Healthy laboratory technicians	22	1	0	3	18
III	Submitted for heterophil studies, all with "nonreactive" blood smears†	250	0	3	53	194
IV	Submitted for heterophil studies; blood smears showed varying degrees of reactive lymphocytosis (>5/100 WBC) but generally short of minimal criteria for IM (see text)	250	1	17	65	167
V	Specimens from a miscellaneous group, including 27 with leukemia/lymphoma, 53 with rheumatoid arthritis, and 19 with "monoclonal" serum peaks	183	5‡	9	42	127
VI	Specimens from a routine serology laboratory, all with detectable cold agglutinins (mononucleosis excluded)	45	2	4	39	0
	Totals	1250	10 (0.8%)	45 (3.6%)	301 (24.1%)	894 (71.5%)

*Cases were categorized as to anti-i, unclassified, or anti-I patterns by comparison of cold agglutinin titers simultaneously performed against cord (i) and adult (I) red blood cell suspensions (see text).

†"Nonreactive" blood smears contained no more than 5 reactive or atypical lymphocytes per 100 leukocytes (i.e., the majority of lymphocytes had a normal appearance with clumped chromatin and smooth cytoplasm).

‡Anti-i was seen in cases of rheumatoid arthritis (1), chronic lymphocytic leukemia (1), nodular lymphoma (1), and paraprotein states (2).

with incubation at either 10° or 16°C. None of the 5 CMV sera demonstrated anti-i.

CA data from control groups and patients with IM and heterophil-antibody-negative mononucleosis-like syndromes are summarized in Table 2.

Absorption studies. Mixtures of 1:10 serum:saline of selected CA sera (3 anti-i, 11 unclassified, and 2 anti-I) were absorbed with 5% suspensions of rhesus monkey cells and then retested against cord and adult cells after a 60-min, 4°C incubation and a 1-min centrifugation. Some sera were also absorbed with suspensions of 10% rhesus monkey or human adult O cells.

When "unclassified" sera were absorbed with rhesus cells, there was a variable loss of anti-i cell reactivity. In 4 of 11 heterophil-positive specimens, titers against cord cells became negative (<20). Such absorption resulted in a small decline in titer against adult cells. Following absorption with human adult O cells, activity against adult O cells was decreased and reactions with cord cells were generally unchanged.

Table 2. Summary of Cold Agglutinins (CA) in Infectious Mononucleosis (IM), Heterophil-negative Mononucleosis-like Illnesses, and Control Groups

Group	n	CA Pattern (%)			
		Anti-i	Unclassified	Anti-I (≥ 1:14)	Negative (≤ 1:7)
Epstein-Barr Virus–IM (7/157 heterophil-negative)	157	31.8*	36.4	19.7	12.1
Cytomegalovirus–IM (29/29 heterophil-negative)	29	0.0	34.5	58.6	6.9
Control groups I–IV	1022	0.3	3.1	21.5	75.1
Control group V	183	2.7	4.9	23.0	69.4

*Includes 12 cases with unclassified CA when studied at *only* 4°C (i.e., anti-i became apparent at 22°C or 37°C).

The initial specimen, received 4 days after onset of illness from patient Frio (mentioned above in discussion of papain treatment), showed higher titers with papainized cells, suggesting CA in the Ii system. Subsequent specimens obtained over the next 19 mo showed equal or lower titers with papainized and neuraminidase-treated (RDE) cells as compared to untreated cells. Thus it seemed probable that although the acute sample had Ii reactivity, there was also an underlying CA with anti-Pr–like activity. Further evidence that the subsequent specimens had anti-Pr was obtained from absorption studies. CA activity was removed by absorption with adult O cells while parallel absorption with papainized and RDE adult cells did not reduce the CA titers. Treatment with both 2-ME and DTT removed the CA activity of the one specimen tested.

DISCUSSION

The present study has shown that the anti-i CA pattern is reasonably specific for EBV-induced IM. Anti-i was seen in only a small percentage (0.8%) of controls (Table 1). The results of EBV-specific diagnostic tests on six of these control sera with anti-i CA indicated either long-past EBV infections or as yet no contact with the virus.

Unclassified CA were seen in 68 of 150 (45.3%) serially studied cases of heterophil-positive IM, 1 of 7 (14.3%) cases of heterophil-negative EBV-induced IM, 10 of 29 (37.3%) cases of the heterophil-negative mononucleosis-like syndrome due to CMV, and 2 of 2 (100%) heterophil-negative cases in this study where the causative agent was not determined. Of 68 serum samples from heterophil-positive IM that were initially categorized as unclassified CA at 4°C, 12 had anti-i specificity when tested at 10°C (3 of 14 cases), 16°C, or 25°C. Furthermore, differential absorption with rhesus and human adult O erythrocytes also suggested anti-i in several other cases.

To date, anti-i has not been detected in our CMV-induced cases; however, elevated CA with equal reactivity against cord and adult cells (i.e., unclassified) were encountered in 10 of 29 CMV-induced cases. The unclassified CA pattern was nondiagnostic and was seen in 3.6% of 1250 control patients. However, it was seen in only 1.2% of acutely ill patients whose blood smears did not reveal some degree of "reactive" lymphocytosis (<5 reactive or atypical lymphocytes per 100 leukocytes). In patients with "reactive" lymphocytosis but lacking the

minimal morphological criteria* for IM, the unclassified pattern was seen in 17 (6.8%) of 250 consecutive cases (Table 1). At the conclusion of this study, 16 of the latter 17 serum samples were rethawed and tested with the CMV-IgM test. Three of these 16 specimens gave strongly positive results in the CMV-IgM test. These cases had never been considered as heterophil-antibody-negative mononucleosis owing to the initial absence of significant* numbers of atypical lymphocytes or because convalescent follow-up specimens could not be obtained for confirmatory viral and heterophil studies. It is well recognized that CA of the unclassified type are frequently encountered in serology laboratories and blood banks and are not specific for IM or any heterophil-negative mononucleosis-like syndromes.[24 26] This pattern is also seen in sera from some patients with cold hemagglutination disease,[27] several types of pathologic disorders including metastatic carcinoma and lymphoma,[28] and healthy normal donors.

The detection of anti-I CA or failure to detect CA does not exclude mononucleosis. In 22 of 38 (57.9%) cases of the heterophil-negative mononucleosis syndrome and in 47 of 150 (31.4%) cases of heterophil-positive IM, either no CA activity or anti-I was found. Furthermore, anti-I at low titers can be demonstrated as a "natural" CA in serum from most people.[25,28]

The mechanism by which viral disease leads to the production of cold reactive antibodies is unknown. Among the possibilities, as reviewed by Roelcke,[29] are that (1) the infective agent stimulates abnormal or forbidden clones, thus leading to the breaking of tolerance; (2) CA are due to sharing of antigens between human erythrocytes and the viral agent (e.g., EBV or CMV); or (3) CA arise in response to antigenic changes in red cells induced by viral action. There is presently no way to decide which, if any, of these possibilities is correct.

Significant hemolytic anemia in both classical EBV-induced IM and the mononucleosis-like syndrome due to CMV is uncommon (about 1 of 200 cases). The mechanism by which hemolytic anemia in IM is produced is obscure; however, a popular thesis suggests that complexes of IgG and IgM lodge on the red blood cell membranes, attract complement, and trigger hemolysis.[30] The smaller component is thought to be a 7S CA and the 19S components are possibly cold-reactive rheumatoid-like factors. A recent study by Wilkinson and associates suggests that the aforementioned complex-induced mechanism may not explain all cases of hemolytic anemia in IM, as IgG-anti-i has not been identified in three cases of heterophil-positive IM with moderately severe hemolysis.[31] In the present study, we have been unable to demonstrate 7S CA in 2-ME inactivated specimens obtained mainly from patients without obvious hemolysis.

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