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Army Medical Research Lab., Fort Knox, Ky. (Report No. 67)

The Role of Cold Hemagglutinins in Frostbite - AMRL Project No. 6-64-12-Z

Weiner, David 12 Oct '61 13pp tables

Cold - Physiological effect
Blood - Coagulation

Medicine (19)
Physiology (2)

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ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 67
12 October 1951

THE ROLE OF COLD HEMAGGLUTININS IN FROSTBITE*

*Subtask under Environmental Physiology, AMRL Project No. 6-64-12-7,
Subtask, Cold Injury Studies.



MEDICAL RESEARCH AND DEVELOPMENT BOARD
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Report No. 67

THE ROLE OF COLD HEMAGGLUTININS IN FROSTBITE*

by

David Weiner, Capt., M.C.

from

Army Medical Research Laboratory
Fort Knox, Kentucky
12 October 1951

* Subtask under Environmental Physiology. AMRL Project No. 6-64-12-Z,
Subtask, Cold Injury Studies.

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Project No. 6-64-12-Z
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12 October 1951

ABSTRACT

THE ROLE OF COLD HEMAGGLUTININS IN FROSTBITE

OBJECT

To determine the relationship between resistance to cold injury and the presence of cold hemagglutinins in the blood.

RESULTS

A. Positive cold hemagglutination was found in 74.8% of 115 frostbite cases as compared to only 40.3% of 308 unselected controls.

B. Titers of 1:8 or higher were found in 25.2% of the frostbite cases as compared to only 6.5% of the controls.

C. Titers in the frostbite group ranged up to 1:64 as compared to only 1:16 in the control group.

D. Titers were higher and more frequent in Negroes than in white men.

E. Experiments performed on 3 human volunteers revealed no cold hemagglutination after severe exposure to cold during which skin temperatures of the digits were as low as -9°C . and in whom first degree frostbite was often produced.

F. Experimental frostbite in rabbits produced no increase in cold agglutinin titer.

CONCLUSIONS

A. The presence of cold hemagglutinins in the blood and the occurrence of frostbite are significantly related.

B. Exposure to cold with the production of frostbite does not appear to produce or increase cold hemagglutinin titer.

C. Final proof is not available but it is inferred that intravascular cold hemagglutination in an extremity exposed to cold may play a part in the pathogenesis of frostbite.

D. The more frequent occurrence of high titers of cold hemagglutinin in Negroes may be a factor in their reportedly (46) greater susceptibility to cold injury.

RECOMMENDATIONS

A. Cold hemagglutinin studies on troops scheduled for cold weather combat duty should be carried out in order further to clarify the relationship to subsequent cases of frostbite.

B. Additional experiments on animals are suggested.

C. Serious consideration should be given to the possibility of reducing the incidence of frostbite in soldiers by avoiding exposure to cold weather of individuals with high titer cold agglutination.

Submitted by:
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THE ROLE OF COLD HEMAGGLUTININS IN PROSTEBITE

I. INTRODUCTION

Little has been added to our understanding of cold hemagglutination since Landsteiner (27) in 1903 gave the first adequate description of the phenomenon in guinea pigs, chickens, horses, dogs and cattle. He demonstrated the dependence of the agglutination on low temperature with dispersion on rewarming and was able to prepare solutions of agglutinin in normal saline solution. He further demonstrated that the agglutinin is contained in the globulin portion of the serum. The first description of the phenomenon in humans was given by Biffi (5) in 1903. Clough and Richter (7) described the agglutinin as being in the euglobulin fraction while Stats, Perlman, Bullowa and Goodkind (41) demonstrated that it has the electrophoretic mobility of gamma globulin and quantitatively determined its antibody nitrogen equivalence.

The serological characteristics distinguishing cold hemagglutination from other types of agglutination have been well established and may be summarized as follows:

- (1) Agglutination of red cells by serum containing cold agglutinin can be demonstrated best between 0° and 5°C, rarely above 25°C and practically never at 37°C.
- (2) The agglutination may be reversed by warming to 37°C and reappears on cooling. This process may be repeated without apparent damage to the red cells.
- (3) Serum can be exhausted by repeated absorption in cold and released by rewarming.
- (4) The agglutinin is not inactivated by heating to 56°C for 30 minutes.
- (5) The agglutinin survives cold storage with only slight decrease in strength for periods ranging from 3 weeks to a year or more.
- (6) The agglutinin is active against human cells of any group and against animal cells of many unrelated species.

The occurrence of cold hemagglutinins in normal healthy humans has not been thoroughly studied. Freedman and Mirsky (15) found cold agglutinins in 14.6% of 103 normal controls, Amzel and Hirzfeld (2) in 4.7% of 238 sera obtained from routine bloods sent to the laboratory for Wasserman tests and Kettel (25) in 95% of 600 patients hospitalized for chronic diseases.

The association of cold hemagglutination with a variety of clinical conditions has been the subject of numerous reports. Comprehensive reviews of the literature were made by Stats and Wasserman (42) and by Platt and Ward (33).

From the literature it appears that cold hemagglutination is found with a high degree of frequency only in primary atypical pneumonia (7, 12, 14, 15, 21, 32, 44) and in trypanosomiasis (6, 10, 24, 47) in neither of which a

pathogenetic relationship has been established. High titers are a constant feature in the former reaching their peak during the second to fourth week of the disease and falling rapidly during convalescence. The occurrence of cold agglutinins in malaria and other parasitic infestations has not been adequately studied.

The presence of cold hemagglutination in various types of acquired hemolytic anemias has been widely reported (3, 9, 17, 20, 31, 37, 43, 45) but the diversity of hematologic pictures and the infrequency of occurrence of cold agglutinins make analysis of the relationship difficult. It is possible that cold agglutinins may play a pathogenetic role in certain types of hemolytic anemias particularly those occurring during the course of primary atypical pneumonia (14, 21, 32, 45).

Paroxysmal cold hemoglobinuria is not infrequently attributed to the presence of marked cold hemagglutination (1, 3, 19, 26, 29, 34, 36, 40). Such cases can be clearly differentiated from the more common type of paroxysmal cold hemoglobinuria associated with syphilis in which the Donath-Landsteiner test is positive and cold hemagglutination usually absent (42). Raynaud's syndrome and similar peripheral vascular phenomena, with or without paroxysmal cold hemoglobinuria, have also been associated with cold hemagglutination (1, 3, 4, 8, 9, 16, 18, 19, 22, 23, 29, 34, 38). Two such cases resulted in gangrene of the tips of the extremities (29, 40). In the case reported by Stats and Bullowa (40) the mechanism was clearly defined by the demonstration of unilateral hemoglobinemia following exposure of one forearm to cold and by the demonstration of hemagglutination, discontinuity of the blood stream and slowing of blood flow in conjunctival vessels following irrigation of the sac with iced saline solution. Stats (39) further showed that cold-agglutinated red cells are easily hemolyzed by gentle shaking.

From the foregoing it is not too difficult to envision a relationship between the presence of cold hemagglutinins and the occurrence of frostbite and other forms of local cold injury. Such a thought, in fact, has not escaped recognition. Parker (30), in a personal communication cited by Platt and Ward (33), stated that cold-hemagglutination may play a part in the production of trenchfoot. Lange, Weiner and Boyd (28) observed intravascular clumping of red cells in tissues exposed to cold and suggested a relationship to cold-agglutinins or other agglutination reactions. Stats and Wasserman (42) suggested that individual tolerance to cold may depend on the titer of normal cold hemagglutinins.

In the present study an attempt is made to demonstrate a positive correlation between the occurrence of cold hemagglutination and the incidence of frostbite in military personnel.

II. EXPERIMENTAL

A. Methods

1. Subjects

a. Frostbite group.

Cold agglutination tests were performed on 115 soldiers who had suffered frostbite of varying degree 6 to 9 months previously during the 1950-51 winter campaign in Korea. Their age and race distribution is shown in Table 1.

TABLE 1

AGE AND RACE OF FROSTBITE SUBJECTS

AGE	WHITE	NEGRO
17 - 20	33	16
21 - 24	25	17
25 - 30	9	6
31 or over	5	4
TOTALS	72	43

b. Control group.

Cold agglutinin studies were carried out on 308 unselected, apparently normal, healthy enlisted men from the Army Medical Research Laboratory and the Third Armored Division at Fort Knox. Approximately half of these men had seen combat service in Korea during the 1950-51 winter. All these men denied ever having suffered frostbite. Their age and race distribution is shown in Table 2.

TABLE 2

AGE AND RACE OF CONTROL SUBJECTS

AGE	WHITE	NEGRO
17 - 20	34	49
21 - 24	72	52
25 - 30	41	16
31 or over	26	18
TOTALS	173	135

c. Experimental cold exposure in humans.

During the course of other experimental work in progress at the Army Medical Research Laboratory three normal young enlisted men were repeatedly exposed to temperatures of -40° to -60°C . In two of these digital skin temperatures as low as -9°C were reported and first degree frostbite was often produced. Repeated cold agglutinin studies were performed on these men.

d. Experimental frostbite in rabbits.

Cold agglutination tests were performed on 13 rabbits before and after the production of experimental frostbite.

2. Procedures*

The method of determining the titer of cold agglutinin differed in no way from the methods reported in detail by nearly every investigator in the field. A few preliminary experiments were performed to confirm the fact that sera may be stored at 5°C for a week or more without diminution in titer (11, 12, 32, 33, 42), that cell suspensions from individual or pooled Group O donors and the subjects own cells (auto-agglutinins) gave almost identical results, and that cell suspensions more than 3 days old were unsatisfactory. The procedures used throughout the present study were as follows:

a. Collection of blood specimens and preparation of sera.

Blood was collected in dry, sterile syringes from an antecubital vein, placed in 15 x 125 mm. test tubes and allowed to clot at 37°C in a water bath or incubator. In a few instances clotting was permitted at room temperature (25°C). After sufficient clot retraction had taken place the blood was centrifuged at 2000 r.p.m. for at least 30 minutes and the serum carefully decanted. In most cases the sera were tested immediately; in a few cases sera were stored at 0° to 5°C for 1 to 3 days prior to titration.

b. Preparation of red cell suspensions.

In most instances cells from a single Group O donor were used. In a few instances pooled cells from several Group O donors were used. Cells were usually obtained by placing 2 to 5 ml. of freshly drawn blood in 50 to 100 ml. of warm, normal saline solution and separating by centrifugation. Occasionally, cells were obtained by washing the blood clot with warm saline solution. In either case the cells were washed at least 3 times by repeated centrifugation and resuspension in warm fresh saline solution. After having been washed the cells were resuspended in sufficient saline solution to make a 1% suspension.

c. Titration.

Serial dilutions of the serum by the doubling method were prepared in 12 x 100 mm. serological tubes so that each tube contained 0.5 ml. of solution. To each tube 0.5 ml. of the red cell suspension was added making the final serum dilution in the first tube 1:2, in the second tube 1:4, etc. The tubes were then shaken gently to assure even dispersion of the red cells and placed in a refrigerator at 0° to 5°C. The tubes were again gently shaken after being in the refrigerator 30 to 60 minutes and immediately replaced to remain over night.

* Cold hemagglutinin determinations were carried out with the technical assistance of Sgt. Glenn Case, USAF, and Pvt. Walter Berg, USA.

d. Reading of agglutination.

After 16 hours in the refrigerator at 0° to 5°C., the tubes were removed a few at a time and read immediately, before rewarming could occur. The tubes were shaken gently to loosen the red cell mass and observed with a two inch magnifying lens in a moderately strong light. The agglutinations were graded in the following manner:

- 4 † massive agglutination of all or nearly all cells.
- 3 † large clumps with clear surrounding fluid.
- 2 † many smaller clumps with pinkness of surrounding fluid.
- 1 † minimal, but definite, agglutination visible.

The titer is reported as the highest dilution in which definite agglutination was visible. All agglutinations were confirmed to be cold agglutinations by observing disappearance on rewarming to 37°C.

B. Results

1. Positive cold hemagglutination was present in 74.8% of the frostbite cases and in 40.3% of the normal controls. Titers, in general, were low as compared to those commonly found in primary atypical pneumonia but ranged up to 1:64 in the frostbite group and 1:16 in the controls. The difference between the two groups is more strikingly apparent when the higher titers are considered. Titers of 1:8 or higher were found in 25.2% of the frostbite cases and only 6.5% of the controls. Table 3 summarizes the results obtained.

TABLE 3
COLD HEMAGGLUTININ TITERS IN FROSTBITE
AND NORMAL SUBJECTS

	FROSTBITE	CONTROLS
Total cases	115	308
Positive	86 (74.8%)	124 (40.3%)
Titers	1:2	41 (35.7%)
	1:4	16 (13.9%)
	1:8	16 (13.9%)
	1:16	7 (6.1%)
	1:32	4 (3.5%)
	1:64	2 (1.7%)
Negative	29 (25.2%)	184 (59.7%)

Analysis of these data by the chi square method and other methods indicated a statistical significance of better than 0.001. Thus, the probability of obtaining such results by pure chance is less than one in a thousand.

2. Positive cold agglutination occurred more frequently in Negroes than in Whites both in the control group and in the frostbite group. Table 4 compares the results obtained in Negroes and in Whites.

TABLE 4

COLD AGGLUTININ TITERS BY RACE IN FROSTBITE AND CONTROL SUBJECTS

		FROSTBITE		CONTROLS	
		White	Negro	White	Negro
Totals		72	43	173	135
Positive		49 (68.0%)	37 (86.1%)	66 (38.2%)	58 (43.0%)
Titers	1:2	26 (36.1%)	15 (34.9%)	29 (16.8%)	26 (19.3%)
	1:4	10 (13.9%)	6 (13.9%)	29 (16.8%)	20 (14.8%)
	1:8	8 (11.1%)	8 (18.6%)	5 (2.9%)	9 (6.7%)
	1:16	4 (5.5%)	3 (7.0%)	3 (1.7%)	3 (2.2%)
	1:32	1 (1.4%)	3 (7.0%)		
	1:64		2 (4.7%)		
Negative		23 (32.0%)	6 (13.9%)	107 (61.8%)	77 (57.0%)

3. Within the limited age range of this series, age seemed to be a definite factor in the occurrence of cold agglutinins; the frequency in general, decreasing with advancing age. Table 5 shows the titers obtained at different ages in the control group.

TABLE 5

THE INFLUENCE OF AGE ON COLD AGGLUTININ TITERS
(Normal Subjects)

		17-20	21-24	25-30	31 of over
Totals		83	124	57	44
Positive		41 (49.4%)	45 (36.3%)	25 (43.9%)	13 (29.6%)
Titers	1:2	22 (26.5%)	19 (15.3%)	8 (14.0%)	6 (13.7%)
	1:4	14 (16.9%)	17 (13.7%)	14 (24.6%)	4 (9.1%)
	1:8	3 (3.6%)	7 (5.7%)	3 (5.3%)	1 (2.3%)
	1:16	2 (2.4%)	2 (1.6%)		2 (4.5%)
Negative		42 (50.6%)	79 (63.7%)	32 (56.1%)	31 (70.4%)

4. In the three normal subjects exposed to experimental cold environment cold agglutination tests were negative both before and after exposure.

5. In rabbits subjected to experimental frostbite cold agglutinin titers after exposure were practically identical to those obtained previous to exposure. Table 6 shows titers obtained in 13 rabbits. Frostbite of varying degree occurred in all animals.

TABLE 6
COLD HEMAGGLUTININ TITERS IN RABBITS SUBJECTED
TO EXPERIMENTAL FROSTBITE

Rabbit	Degree of Frostbite	TITERS			
		Before Exposure	1 Day After Exposure	8 Days After Exposure	15 Days After Exposure
B	First	1:8	1:16	1:16	
C	First	1:8	1:8		
D	Third	1:16	1:16		
E	First	1:32	1:32	1:16	1:8
H	Fourth	1:64	1:32		
J	Fourth	1:32	1:32		
L	Fourth	1:16		1:8	
M	Fourth	1:32		1:64	
N	Third	1:8	1:8	1:8	
P	Fourth	1:16	1:16		
S	Fourth	1:16	1:16		
W	Fourth	1:16	1:16	1:16	1:8
X	Fourth	1:16	1:16	1:16	

III. DISCUSSION

There seems little doubt that a definite correlation exists between the occurrence of frostbite in exposed individuals and the presence of demonstrable cold hemagglutinins in the blood. This statement seems warranted in spite of the 6 to 9 month interval between the frostbite episode and the laboratory test, there being no reason to suppose that the

control subjects did not have equal opportunities to acquire cold agglutinins during this period. The incidence of primary atypical pneumonia and other diseases associated with cold agglutinins was negligible in both groups.

The pathogenetic relationship of cold agglutinins to frostbite, on the other hand, is not conclusively proved. However, while the data are admittedly scant, the failure of appearance of cold agglutinins in the blood of the humans exposed to cold and the lack of rise of titer in the rabbits subjected to frostbite indicate that the reverse relationship does not exist. Thus, in an individual exposed to cold the presence of cold hemagglutination may cause intravascular clumping in the superficial vessels resulting in slowing of the blood flow and, perhaps, occlusion of small vessels. Intravascular clumping has actually been observed by Stats and Bullowa (40) and by Lange, Weiner and Boyd (28) in cold exposure.

It is, of course, obvious that frostbite occurs in the absence of cold agglutination while many individuals with high titers escape injury in spite of apparently severe exposure. This may be due to the multiplicity of factors involved in the production of clinical cold injury among which may be mentioned (1) degree of cold, (2) duration of exposure, (3) degree of moisture, (4) immobility, (5) nutritional status, (6) physical fatigue or exhaustion, and (7) the presence of peripheral vascular disease. Thus, when some or all of these factors combine to produce unusually severe conditions the absence of cold agglutinins will not save the individual from irreversible injury. Conversely, under milder conditions an individual may escape injury in spite of the presence of marked cold agglutination. Conceivably, there may be a broad mid-zone of conditions under which the presence or absence of cold agglutination may play a decisive role.

IV. CONCLUSIONS

A. A statistically significant relationship exists between the occurrence of frostbite and the presence of demonstrable cold hemagglutinins in the blood.

B. The available evidence indicates that intravascular cold hemagglutination in an extremity exposed to cold may play a pathogenetic role in frostbite.

C. The higher incidence of frostbite in Negroes (46) may be partially due to the more frequent occurrence of cold agglutinins in this race.

V. RECOMMENDATIONS

Additional data on the occurrence of cold hemagglutination in frostbite victims should be accumulated as subjects become available. The occurrence and persistence of cold agglutinins in normal subjects should be intensively and extensively studied. Field studies on troops slated for cold weather combat duty are urgently recommended. Experimental work on animals may be a valuable method of confirming the pathogenetic relationship herein suggested.

Serious consideration should be given to the possibility of reducing the incidence of frostbite in soldiers by avoiding exposure to cold weather of individuals with high titer cold agglutination.

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