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Research Article

COLD AGGLUTININS. V. DETERIORATION OF COLD ISOHEMAGGLUTININS ON STORAGE

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When the first instances of marked cold hemagglutination were noted in this laboratory in cases of primary atypical pneumonia of unknown etiology (1), a number of sera were available from other similar cases which had been studied during the preceding months. Most of these sera had been obtained from cases in this hospital but many were from other hospitals. The large majority of them gave negative results when tested for cold agglutinins. On the other hand, sera from characteristic cases obtained at the proper time in the course of the disease from patients who were being currently observed gave positive results.

Several possible explanations for this discrepancy were considered. In the first place, it was possible that the earlier cases differed from the current ones with respect to the occurrence of cold agglutinins. This explanation seemed unlikely in view of the similarity of the clinical course in both groups of cases. It seemed possible, however, that variations in severity of the cases might have accounted for the differences. The earliest cases in which the cold agglutinins were observed were very severe ones and many of the others that were being encountered at that time seemed to be of greater severity than those which had been described from schools and from military hospitals. Many of the sera which had been stored were from mild cases in young adults and some of them were from military hospitals. Interestingly enough, most of the few positive results among the stored sera were from very severe cases in which there was extensive pulmonary involvement (2).

Another possible explanation depended on the likelihood that most of the sera that were stored had been improperly prepared and preserved. It is usually more convenient to collect a number of specimens of blood, store them in a refrigerator over night, and take off the serum on the following day. The possibility must, therefore, be considered that, without special precautions having been taken to warm the bloods, some at least of the cold agglutinins might have been adsorbed on the red blood cells of the clot and left behind in the process of taking off the serum. Such a loss can readily be demonstrated, particularly if the blood remains cold while the serum is rapidly removed. For the most part, however, the bloods were kept at room temperature for some time before the sera were separated. Subsequent observations have indicated that under the latter circumstances very little drop in the titer of cold agglutinins occurs.

Finally, the possibility was considered that the cold agglutinins had deteriorated on storage. This was suggested by other workers (3) to explain the low titers of some of the stored sera from characteristic cases of atypical pneumonia in their series. In another study (4), more than one-third of the sera had been stored for several months although it is not stated what proportion of the negative sera in cases of atypical pneumonia were among those which had been stored. Alternative explanations were given by the latter authors for most of the negative results which they obtained in their cases of primary atypical pneumonia.

The present paper deals with a study which was directed specifically towards determining the effect on the titer of cold isohemagglutinins of storage for varying periods up to 18 months in an ordinary electric refrigerator, and for shorter periods at room temperature.

MATERIALS AND METHODS

Almost all of the sera in this study were obtained at various stages of the disease from the patients with characteristic primary atypical pneumonia of unknown etiology, who were included in the previous study (2). A few sera which were obtained from 3 of the patients with acute hemolytic anemia (5) are also included.

The bloods were all obtained and handled with sterile precautions. In many instances, the sera were separated at room temperature (20 to 25° C.) but most of them were warmed to 37° C. before the serum was taken off.

Special care was taken to obtain serum free from cells. The sera were then stored in sterile rubber stoppered test tubes in an ordinary household refrigerator in which the temperature varied between 5 and 10° C. Many of these sera were also used for a variety of other tests which were carried out at the same or at different times. These sera were allowed to come to room temperature before each of the tests was carried out and were often kept at that temperature for several hours while the tests were being set up. Toward the end of this study, due to unavoidable circumstances, all of the sera were kept at room temperature for 3 to 6 weeks. The final tests were done after this period.

The method used for determining the titer of cold agglutinins was described in a previous paper (5). The results recorded in the present study are based upon tests carried out with a single method. Briefly, the tests were set up as follows: to 0.5 ml. of serial 2-fold dilutions of serum were added equal volumes of a 2 per cent suspension of 2- to 4-day-old group O red blood cells from a single donor. The mixture was shaken and kept in a refrigerator over night. The tests were then read, usually after the racks were placed in an ice bath. The agglutinations in all positive tests were completely dispersed at 37° C. The initial tests on each of these sera were carried out either on the day when the blood was obtained or within 3 or 4 days. The tests were repeated on large batches of sera at irregular intervals, either in connection with other tests of the same sera or when otherwise convenient. The titers are expressed as the reciprocal of the greatest *final* dilution of serum that gave definite (1+), coarsely granular, or floccular agglutination which was visible without magnification.

RESULTS

A total of 753 tests were done on the 248 sera, or an average of 3 tests of each serum. The original titers varied from 10 to 5120. In 19 of the sera, one additional test was done after the titer had dropped below 10. The results obtained in the last test of these sera are not included in the analysis since they only confirmed the results of the previous one. Excluding these 19, there were 486 repeat test of the 248 sera and these formed the basis of the analysis which follows.

The outstanding feature of the results of the repeated tests was the irregularity and unpredictability of the rate of deterioration of the cold agglutinin titers. To be sure, some of the variations may be due to the different conditions to which some of the sera might have been subjected inadvertently. Many of these variations are not measurable since, in a general way, all of the sera were handled in the same manner.

In a few specimens, contaminants were encountered, particularly in the form of molds or putrifying bacteria. The variations from the previous levels in such sera were not strikingly different from those observed in sera which had remained sterile throughout. In fact, some of the most heavily contaminated ones retained their original titers for several months after the contaminants first appeared and without special treatment of the serum in the interim. In others, however, there were varying degrees of hemolysis of the red blood cells which resulted from the contaminants and which obscured the end-points.

Considering the number of variables involved and the difficulty of measuring most of them, the total number of sera tested is obviously inadequate for the purpose of defining accurately the rate of deterioration of the cold agglutinin titers in relation to all of the factors involved. One feature which seemed to be important in determining the persistence of the cold agglutinins was the intensity of the original reaction and this was, in general, proportional to the titer of the serum. The results are, therefore, considered in relation to the titers obtained in the initial and subsequent tests. Unfortunately, circumstances did not permit complete studies of all of the sera at the same intervals or at frequent and regular intervals.

A few points require comment. In the first place, some of the sera were exhausted earlier than others. In addition, some of them lost their agglutinating properties earlier than others. The numbers of sera in which this took place naturally accumulated with time and the deteriorated sera were not retested after the titer dropped below 10, except as already mentioned. The accumulated results of the later tests, therefore, appear to denote less deterioration than was actually the case.

Titers higher than the original ones were obtained in a number of sera that were retested at varying intervals even after 16 months. Most of these higher titers were observed between 7 and 15 months after the initial tests. Two possible explanations may be offered for these results. Evaporation may conceivably account for the concentration of the antibodies in some of the sera. Few, if any of the sera, however, showed sufficient evaporation to account for the observed 2- or 4-fold increases in titer on the basis of that factor alone. Another plausible explanation for the higher titers depends upon the errors involved

in the tests. The higher titers in the later tests were obtained mostly in sera which initially had the lowest titers and the frequency with which these apparent increases were observed diminished steadily as the original titers increased. Thus, about one-third of the tests which were repeated on sera which initially had titers of 10 or 20 showed 2- or 4-fold higher titers:—about 1 in 4 of the sera with initial titers of 40; 1 in 6 of those which originally had a titer of 80; and about 1 in 10 of those with original titers of 160 or 320 had higher titers when retested. Among the sera in which the original titer was 640 or higher, none was found to have a greater titer when subsequently retested. This correlation seems to be quite regular and probably is significant. It may depend, in part at least, on the fact that the end-points were more sharply defined in sera of high titers and less well defined in those of low titers so that errors in reading the original and subsequent titers might be limited mainly to the sera of low titer. Some of these discrepancies may also depend on the use of cells from different donors.

No other uniform or obvious trend could be discerned. A study of the individual sera in which 3 or more tests were done in the course of this study likewise revealed no uniformity. In some of these sera, progressively lower titers were obtained in each successive test. In others, there was an early drop in the titers which then remained stationary and occasionally even showed apparent increases of 2 or 4-fold over the results of the preceding tests. Still other sera showed higher titers in the second or third tests, or in both, and subsequent ones showed a drop in titer.

The same variations were also noted with respect to the changes in titer resulting from exposure of the sera to room temperature. In general, a larger proportion of the latter tests showed a drop in titer, and the reductions were, on the whole, considerably greater than those observed over much longer periods in the same sera before they had been exposed to room temperatures or in other sera which had not been exposed to the higher temperatures. Nevertheless, the same general changes were noted, that is, some sera showed marked drops, others only moderate drops in titer, and still others showed no change or an apparent increase in titer in spite of the exposure to room temperature.

These wide variations and the relatively small number of observations have made it difficult to define the pattern of the deterioration of the cold agglutinin titers. A few correlations were made and while they are of some interest, most of them are not very revealing. In Table I, there are listed the numbers of sera showing changes of various extent from the original cold agglutinin titers, arranged according to the time elapsed after they were first tested. The outstanding feature revealed in this table is the relatively large proportion of the older sera which showed large decreases in titer or loss of cold agglutinins after exposure to room temperature. No striking progression of the deterioration can be discerned from these results. In this table, of course, the height of the original titer is not taken into consideration.

TABLE I

Deterioration of cold isohemagglutinins on storage

Change from initial titer	Months after initial determination					Totals
	4 or less	5 to 6	7 to 9	10 to 15	16 or more	
2 or 4-fold increase	2	3(2)	25(4)	23(1)	9(9)	62(16)
No change	5	8	40(7)	31	7(7)	91(14)
2-fold decrease	5	11(3)	37(7)	21(2)	18(18)	92(30)
4-fold decrease	6	6(1)	20(4)	21(2)	12(12)	65(19)
8-fold decrease	4	7(2)	10(1)	12(2)	18(18)	51(23)
16-fold or greater decrease	1	5	7(1)	12(6)	13(13)	38(20)
Decrease to <10 *	2	11(8)	17(7)	30(16)	27(27)	87(58)
Totals	25	51(16)	156(31)	150(29)	104(104)	486(180)

* Not included above.

Numbers in parentheses refer to sera which had been kept at room temperature (20 to 25° C.) for 3 to 6 weeks prior to testing.

TABLE II

Time of disappearance of cold isohemagglutinins in relation to original titer

Initial titer	Months after initial determination when titer first decreased to <10						No. of sera tested	Percentage of sera decreased to <10
	4 or less	5 to 6	7 to 9	10 to 15	16 or more	Total		
10		2(1)		3(1)	2(2)	7(4)	11	64
20	1	2(2)	5(2)	3(2)	5(5)	16(11)	30	53
40	1	2(1)	3	9(4)	5(5)	20(10)	33	61
80		3(2)	4(2)	4(1)	2(2)	13(7)	35	37
160		2(2)	2(2)	7(4)	5(5)	16(13)	46	35
320			1	3(3)	8(8)	12(11)	55	22
640			2(1)	1(1)		3(2)	19	16
1280 to 5120						0	19	0
Total <10	2	11(8)	17(7)	30(16)	27(27)	87(58)	248	35
Number of sera tested	25	51(16)	156(31)	150(29)	104(104)	248*		
Percentage of sera decreased to <10	8	19(50)	11(23)	20(55)	26(26)	35		

Numbers in parentheses refer to sera which had been kept at room temperature (20 to 25° C.) for 3 to 6 weeks prior to testing.

* The apparent discrepancy between this total and the sum of the numbers to the left of it in the same horizontal line is due to the fact that many sera are listed in more than one of the vertical columns.

The time of essential disappearance of the cold agglutinating properties of the sera is correlated with the original titers in Table II. As the original titer increased, there was a steady drop in the proportion of sera in which the titer was observed to drop below 10. This was, of course, to be expected. Thus, all of the sera in which the original titer was 1280 or higher retained some of the cold agglutinins throughout the period of study, whereas more than one-half of the sera in which the original titer was 40 or less, had dropped to a level below 10 in the same period and an intermediate number of those with original titers of 80 to 640 had such a drop.

The proportion of all the cases in which a reduction in titer occurred showed no uniform trend in relation to the length of time they were stored. This is due in part to the irregular proportion of cases of different titers which were examined at the different intervals. Furthermore, no allowance is made in this or in the preceding table for sera in which the titers had previously dropped below 10 and therefore were not included in the subsequent tests. Their inclusion would, of course, tend to increase the proportion of sera showing deterioration at successive intervals.

An attempt was made to find an expression for the deterioration in titers of cold agglutinins that would include most of the important factors involved. It was necessary to take into account the original titers, the wide variation in the rate of deterioration from these titers that occurred in different sera, and the fact that changes in titer occurred in geometric proportions. Any expression of the changes should tend to minimize the observed increases and give proportionately greater weight to the larger variations. If the original titer is represented as 5×2^n and subsequent titers are indicated as 5×2^m, the ratio of the sums of the exponents m and n would satisfy most of these qualifications. The ratio would represent an average and provide a fair approximation of the proportion of the original titers which persist. In order to express the extent of deterioration, this ratio readily may be converted to express the percentage drop from the original titer by applying the following formula:

$$\text{Index of deterioration } (D) = \left(1 - \frac{\Sigma m}{\Sigma n}\right) \times 100.$$

This index has been calculated for all the sera tested at various intervals and also for the results of all of the tests done on sera which originally had the same titer. The results are shown in Table III. The effect of the exposure to room temperature is also shown in this table and was obtained by calculating separately the index of deterioration in those sera which had been so exposed.

TABLE III

Estimation of the deterioration of cold isohemagglutinin titers

According to:	Index of deterioration *		
	A. All sera	B. Sera stored 3 to 6 weeks at 20 to 25° C.	C. All sera excluding those listed in B
Months of storage:			
4 or less	29		29
5 to 6	34	54	28
7 to 9	21	29	19
10 to 15	33	67	24
16 or more	49	49	
Original titers:			
10	15	100	−38†
20	20	53	−10†
40	41	53	35
80	36	50	25
160	33	54	23
320	29	47	19
640	32	38	29
1280	33	45	25
2560	37	46	32
5120	32	44	25
Total—All sera	32	48	23

* Index of deterioration (D) is calculated according to the formula:

$$D = \left(1 - \frac{\Sigma m}{\Sigma n}\right) \times 100$$

the original titer of the serum having the value 5×2^n and each of the values subsequently observed being given the value 5×2^m. When the titer is <10, m is given a value of 0 for present purposes.

† Titers were higher than the original ones.

Note: The table showing all of the data upon which these numbers are based has been omitted to conserve space.

From this table, it is seen first of all that deterioration in the cold agglutinin titers was greatly accelerated by exposure to room temperature. Roughly speaking, there was approximately the same degree of deterioration during the 3 to 6 weeks when the sera were exposed to room temperature as took place during the entire preceding period of several months when the sera were kept in the refrigerator. This is also evident from the percentages shown at the foot of the columns in Table II.

From the manner in which these data were obtained, it was not possible to demonstrate the progression of the deterioration with time. This has already been alluded to previously. The index of deterioration, as shown in Table III, does not appear to be greater after different periods of storage except as this was influenced by the exposure to room temperature. When, however, account is taken of the progressive number of sera which had deteriorated to a titer below 10 at each successive interval, the index would tend to increase with time as might be expected. The effect of the height of the original titer on the deterioration is also indicated in Table III. Among the groups of sera which originally had titers of 10 or 20, there appears to be an increase rather than a decrease in titer. This is probably more apparent than real. The number of observations is small and, as already mentioned, the greatest errors are probably involved in the titrations of the sera which have such low titers. In all other sera, the index of deterioration was essentially the same and seemed to be independent of the original titer.

DISCUSSION

From the data presented, it is seen first of all that appreciable deterioration of cold agglutinin titers takes place with the usual type of storage of sera in an ordinary electric refrigerator over a period of several months. No uniform rate of deterioration was noted in these sera and wide variations occurred. In sera which had low or moderate titers, a large proportion retained their original titers or even showed apparent increases. Such increases over the original were not observed among the sera which originally had very high titers. Decreases in titer of varying extent were observed among each of the various groups of sera that were tested.

The results of the tests in sera exposed for a relatively brief period at room temperature showed similar variation. The deterioration, however, was more marked and occurred more regularly when compared with the changes observed after the longer periods of storage at 5 to 10° C.

It is to be borne in mind that the sera on which this study is based were originally collected specifically for the purpose of determining cold agglutinin titers. Precautions were taken to avoid adsorption of agglutinins from these sera before they were separated and stored. Some data were obtained which indicated that some reduction in titer may take place if care is not taken to separate the serum at temperatures ranging between 20 and 37° C. Only slight reductions in titer occasionally occur in sera of low or moderate titer

which are separated at room temperature instead of at 37° C. Greater or significant losses can be demonstrated only in sera of very high titers (6, 7).

Some evidence has also been obtained to indicate that the original cold agglutinin titers may be completely preserved by lyophilizing the serum. Through the courtesy of Dr. T. Hale Ham, a serum obtained from a patient with acute hemolytic anemia in 1939 was tested for cold agglutinins after it had been lyophilized and kept at room temperature for 4½ years. The titer at the end of this period was identical with that obtained before lyophilization. After the serum was reconstituted, it deteriorated to a moderate extent during several months of storage in a refrigerator and markedly after it had been left at room temperature.

It is not unlikely that the negative results of the tests for cold agglutinins in the sera collected from patients with primary atypical pneumonia during the 1941–42 season and tested more than a year later were the result of a combination of factors. These include: (*1*) the failure to observe proper precautions in order to avoid the loss of cold agglutinins during the collection and separation of the sera, (*2*) deterioration of the cold agglutinins on storage, and (*3*) the original low titers of the sera. The latter, in turn, may have been due to the fact that most of the cases from which those sera were obtained were relatively mild (2). These factors must be taken into account in evaluating the quantitative results based on stored sera.

Because of the wide variations observed and the relatively small number of sera and tests, it has been difficult to express the extent and rate of deterioration accurately. The proposed index of deterioration is an arbitrary expression intended to convey roughly the amount of drop in titer in terms of a percentage of the original titer. It is, necessarily, only a rough approximation of the amount of deterioration and takes into account several significant factors but not all of them. For example, there is no allowance made in this formula for variations in the accuracy of the test. It will, however, serve to point up the need for some simple method of measuring and expressing the deterioration in the biologic properties of sera. For more accurate estimation of the rates of deterioration, it would be desirable to have large numbers of sera and test them all at the same regular intervals. Clinical material is difficult to obtain and to handle in such a manner.

According to the formula presented, there is essentially the same degree of deterioration in all sera, irrespective of their original titers. Obviously, cold agglutinin titers below some arbitrary one, such as 10 or 20, that is considered to be the lowest significant titer, will be reached at different times depending on the original titer. Likewise, among the sera of any given age, the proportions which deteriorated to a level below the significant titer will increase in inverse proportion to the height of the original titer. In the present study, unfortunately, the same sera are not represented in each of the intervals so that such a progression is not indicated.

SUMMARY AND CONCLUSIONS

The titer of cold isohemagglutinins was determined in 248 sera at the time they were obtained and at irregular intervals during the ensuing 18 months. The original titers of these sera ranged from 10 to 5120. The sera were stored throughout this period in an ordinary electric refrigerator which had a temperature range from 5 to 10° C. Many of them were kept at room temperature (20 to 25° C.) during the last 3 to 6 weeks before the final tests were done.

A large proportion of the sera showed significant deterioration in their cold isohemagglutinin titers after storage at refrigerator temperature for several months. The rate and extent of the deterioration varied markedly in different sera. Many of those which originally had low or moderate titers retained those titers unchanged or showed an apparent increase even after more than a year of storage. Others showed decreases in titer of varying extent and at different intervals.

Deterioration of cold agglutinin titers occurred with greater regularity and at a much more rapid rate after the sera were allowed to remain at room temperature.

The method of collecting sera and the factor of deterioration must be considered in the interpretation of the results of tests for cold agglutinins that are done on stored sera.

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