"A"-Antigen Variation in Pigeon Erythrocytes

I. Effect of X-Irradiation on the "A"-Inagglutinable Cell Frequency

By JOHN C. HIERHOLZER, S. L. SCHEINBERG AND P. A. HANSEN

MANY EXPLANATIONS have been offered for the free-cell phenomenon often observed in hemagglutination procedures.1-4 One of the first proposals concerning these cells was that they were young cells which lacked their full complement of agglutininogen.5 Evidence against this hypothesis was provided many years later by Callender et al. and Young et al., who showed that young, nucleated red cells were as agglutinable in specific sera as were adult cells.6,7 Additional evidence on the relation of cell age to inagglutinability is presented in some of the experiments described herein.

Several hypotheses on the origin of inagglutinable red blood cells suffered from the lack of a method capable of detecting very low frequencies of free cells. For this purpose a sensitive isotope dilution method was developed by Atwood and Scheinberg in 1958.8 These authors postulated that genetic heterogeneity existed in any population of cells of sufficient numbers—for example, erythrocytes—and that the exceptional red cells in such a case would be the result of somatic mutation in the erythrocyte precursor cells. They found cells in type A and AB human bloods which apparently lacked the "A"-agglutininogen. These exceptional cells were shown not to be generally devoid of active sites since they did react with specific anti-H, anti-B, anti-M, and anti-N sera as did the unselected cells. This cellular heterogeneity has been termed "erythrocyte automasicism"9 to distinguish it from chimerism (see review by Cotterman9).

X-rays have been widely used to produce mutations. If the "A"-inagglutinable red cells arise as a result of somatic mutation, then x-irradiation should increase the frequency of these cells. Irradiation experiments were therefore carried out with pigeons, and the present paper provides additional evidence on the ability of x-rays to increase the frequency of the "A"-inagglutinable cells. The variation encountered in the response of pigeons to irradiation in this

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study suggests heterogeneity among the inagglutinable cells and among the pigeons. This question is considered in this paper and a companion paper.

METHODS

Agglutinin

The agglutinin used was an extract* of the lima bean, Phaseolus lunatus var. Thorogreen. The "A" antigen of pigeons has been equated to the A of man by absorption studies and by failure of pigeon cells to react with Dolichos biflorus lectin, a strong anti-A reagent.10

All extracts were standardized with respect to hemagglutinating activity: the red cells of a large number of pigeons were tested with dilutions of the new extract and carefully compared with identical titrations using previous extracts. The standardized preparation was stored in convenient aliquots at −34 C. The term "lectin" will be used for the phytohemagglutinin preparation, as suggested by Boyd.11

Agglutinogen "Titration"

Serial twofold dilutions of lectin were used to establish both the sensitivity of pigeon red blood cells (RBC) and the standardization of subsequent lectin preparations. Four drops of lectin dilution were mixed with 2 drops of 2 per cent red cells, incubated at room temperature for 5 minutes, shaken briefly, and centrifuged for 1 minute at 1650 g to assure cell contact. The tubes were then scored for agglutination by gently tapping the bottom to dislodge the pellet. The titer or end-point of agglutination was the last tube (highest dilution of lectin) showing microscopic clumping as observed under 12x magnification on a dissecting microscope. For instance, if this tube contained the 1:1024 dilution of lectin, the cells were said to have a "titer" of 1024.

X-Irradiation

Irradiation of the test pigeons was accomplished with a 200 k.v.p. Picker x-ray machine. The inherent filter of the machine consisted of 2 mm. glass in the x-ray tube, 2 mm. glass in the aperture window, an oil film of 5 mm. thickness, 1.5 mm. bakelite in the extension cone end, and 0.25 mm. Cu + 1.0 mm. Al in the ionization chamber. Additional filters were not used. The enclosed portion, from the tube window to the face plate of the machine, was 28.0 cm.

The pigeon to be irradiated was secured in a motor-driven lucite cylinder revolving at 7.4 r.p.m. and centered 13.0 cm. from the face plate. These conditions provided whole-body irradiation, uniformly administered while the bird was being turned. The machine was operated at peak voltage (200 k.v.p.) and 25.0 ma. for the period of time necessary for the desired dose. The x-ray dose rate was determined from readings of a 100-r thimble ionization chamber† placed in the center of the abdomen of a dead pigeon used as a phantom. The x-ray machine was thus calibrated each day it was used.

Radioactive Tagging with Cr51

Pigeons to be tested for the inagglutinable cell frequency (ICF) were bled from the alar (wing) vein by scalpel-blade venipuncture. Three to 4 ml. of blood were withdrawn into 10 ml. of Alsever's anticoagulant solution. The red cells were washed and labeled with 450 μc. of sodium radiochromate† per millimeter of packed RBC at 35 C. for 30 minutes. The cells were then washed 4 times in cold saline to remove all traces of extracellular label. The washed, labeled cells were diluted and sampled for initial total specific activity.

*Details of the extraction and clarification procedure are available in the Supplement.
†Victoreen total-dose r-meter, Glasser-Seitz Model 70.
†"Rachromate", Na,CrO₄, Abbott Laboratories, Oak Ridge, Tenn.
Assay of Radioactivity

Samples were assayed for radioactivity in a Packard automatic counting system composed of a Model 500D sample changer with a well-type counting chamber and a 2-inch NaI (thallium-activated) crystal, and a Model 314 E dual-channel “Tri-Carb” spectrometer operated at 998 volts with a 10 per cent window. Counting efficiency of the system was 2.3 per cent. The level of count at the end of most experiments was at least 5 times the background count of 18-23 c.p.m., but occasionally was only twice the background. The standard error of the counting procedure was ±2 per cent.

Determination of the Inagglutinable Cell Frequency (ICF)

The inagglutinable cell frequency (ICF) was determined on the Cr\(^{51}\)-tagged red cells by the isotope dilution method reported by Atwood and Scheinberg,\(^{12}\) except that all steps were carried out at a constant room temperature of 22°C. Usually, 10-14 stages were required to achieve the “plateau”—the end of the experiment which was observed as the stage at which all cells capable of agglutinating had done so. When the plateau was reached, successive stages did not result in further losses of radioactivity. As plotted on semilog paper, the average of values on the plateau was the frequency of inagglutinable cells. The decision as to which points constituted the plateau was made by visual inspection of the isotope dilution curve. Rarely was it questionable at which stage the plateau began; in doubtful cases it was found that the inclusion or exclusion of the dubious starting point made no significant change in the average of the other 4 or 5 points on the plateau.

To avoid possible sources of error in the ICF determination, a single lectin preparation was used for an entire experiment, and excess lectin thawed for use on one day was not reused later in that experiment. Another potential source of error was circumvented by using the same human A, carrier cells for every ICF determination on any one bird. The carrier cells were kept in their collecting bag and were not more than 5 days old when used.

RESULTS

Effect of X-Irradiation on the Frequency of Inagglutinable Cells in C. livia

In a preliminary experiment birds heterogeneous with respect to sex, age, breed, and “A”-agglutinogen titer were divided into 5 groups and given different doses of whole-body x-irradiation. The inagglutinable cell frequencies were determined immediately prior to irradiation and at 2- and 4-month intervals afterward. The results\(^{†}\) were highly variable but suggested that there was an increase in numbers of inagglutinable cells at 360 r but decreases in the number of inagglutinable cells at higher doses.

To gain greater precision among the irradiated birds, controls were exercised over a number of factors suspected of contributing to the variability in response to x-irradiation. The 360 r dose of irradiation was chosen since it appeared to provide for the greatest increase in the frequency of “A”-inagglutinable cells. Birds with the same agglutinogen titer were selected since this was suspected as being one of the variables. Birds with a titer of 512 were used because the sensitivity of the isotope dilution method is greater where the initial level of

\(^*\)An outline of this method is available in the Supplement.

\(^{†}\)A summary and the ICF assays are provided in the Supplement.
inagglutinable cells is low—for example, $10^{-4}$ to $10^{-5}$. That is, a small change in the ICF is more evident when the preirradiation level is 1 per 10,000 than when it is 1 per 100. Birds were also more uniform with respect to breed and age than in previous experiments. Finally, the birds were segregated according to sex.

The results are summarized in Table 1 and the detailed data on each bird is available in the Supplement. An example of a series of isotope dilution curves is seen in Figure 1. As in previous experiments the controls were uniformly unresponsive and exhibited little variability. The inagglutinable frequency in the irradiated birds was significantly increased over that of the controls both at 2 months and at 4 months (Fig. 2), and the variation in response was considerably reduced over that observed in previous experiments. The reduced variation in response among the males suggests this group as the subjects of choice for many experiments. Although there is an apparent difference in response between the sexes, with the females seeming to have the greater response, the variation among individual females prevents an accurate determination of the sex effect with the number of pigeons used. Individual differences among the birds, in addition to breed, age, titer, or sex, are undoubtedly responsible for the variation in response to irradiation. This is evident in a consideration of birds which apparently failed to respond to the effects of irradiation.

Three birds from the above experiment failed to respond at 2 months post-irradiation and were given a second dose of 360 r at the 2-month interval (Table 2). They subsequently showed a decrease in the number of inagglutinable cells, followed by a rise at 8 months postirradiation to nearly the base level (Fig. 3).

These experiments demonstrate an effect of x-irradiation even though increases in the inagglutinable cell frequency were not observed. The results indicate that the observed variation in radiation response is primarily of a biological nature and is not related to the procedures involved in radiation or in the isotope dilution method. As in the experiments with high doses of irradiation, the effect is to decrease the number of inagglutinable cells in some of the birds. The possibility that selection may be operative is suggested. It is also clear that the kind of response which is typified by the three birds used in the experiment plays a substantial role in the individual variability in response to irradiation.

Isolation and Testing of Young Erythrocytes

A question which has been repeatedly raised regarding the inagglutinable cells is whether cell age is correlated with the inagglutinable cell frequency. Young, circulating erythrocytes were isolated and their ICF determinations compared with that of the total, unselected cell population. Human A2 cells were chosen for this experiment so as to obtain sufficiently large initial populations to permit isolation of the young cells.

It has been shown with metabolically-labeled cells that a normal population of erythrocytes can be fractionated according to age by serial osmotic hemo-
A-ANTIGEN VARIATION IN PIGEON ERYTHROCYTES. I.

Table 1.—Effect of Sex on Response to Irradiation with 360 r

<table>
<thead>
<tr>
<th></th>
<th>Age (yrs.)</th>
<th>Breed</th>
<th>Titer</th>
<th>Average Factor Change in ICF, with Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>11</td>
<td>1.2</td>
<td>WC, WK</td>
<td>512</td>
</tr>
<tr>
<td>controls</td>
<td>4</td>
<td>1.2</td>
<td>WC, WK</td>
<td>512</td>
</tr>
<tr>
<td>II.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>12</td>
<td>1.1</td>
<td>WC, WK</td>
<td>512</td>
</tr>
<tr>
<td>controls</td>
<td>5</td>
<td>0.7</td>
<td>WC, WK</td>
<td>512</td>
</tr>
</tbody>
</table>

*Breeds of the pigeon *Columbia livia*. WC = White Carneau; WK = White King.

lysis. The youngest cells are the least fragile—that is, most resistant to osmotic shock. In addition, Borun found that young cells are lighter in weight than old cells and are generally more stable.

Various osmotic-shock methods of isolating the young cells from a normal population of human A2 erythrocytes were explored. The concentration of cells before osmotic shock, the final salt concentration during incubation, the incubation time, and the washing time were the principal factors studied.

The isolation method decided upon was the following. A concentrated suspension of washed RBC was added instantaneously to 0.046 M NaCl so that the final salt concentration was 0.050 M and the final cell concentration 7.5 per cent. This mixture was gently stirred for exactly 2 minutes, after which time the lytic reaction was quenched with 2 per cent NaCl to restore isotonic conditions. The cells which survived the single osmotic shock, presumably the youngest cells, were harvested by centrifugation to differentially remove the ghosts.

Using this procedure, the young cells from a unit of human A2 blood were isolated and then compared to the total cell population. Both cell types—that is, the isolated young cells and the total unselected cells—were titrated in the usual manner, and the ICF's were determined by the isotope dilution method using fresh A1 carrier cells. The experiment was repeated with a different A2 blood and A1 carrier.

No significant differences in titer or ICF were found between human red cells resistant to hypotonic shock and the total red cell population from which these young cells were isolated. The factor increases in the ICF of the young cells over the total cells were 1.22 (1.61 x 10⁻² to 1.95 x 10⁻²) and 1.53 (3.69 x 10⁻³ to 5.66 x 10⁻³) for the two experiments. These changes are not significant because they are within the error of the isotope dilution method. (The average and standard error in ICF observed when retesting the same untreated blood is +0.09 ± 0.26, with a range of ± 1.51.) The titers of the two cell types were the same and remained the same for up to 4 days after isolation. This provides additional evidence of the similarity of the two populations of cells. It also provides evidence that the stability of the isolated young cells was not impaired by the isolation procedure. The recovered young cells constituted about 0.43 per cent of the total cell population.

To determine the possible error in the ICF determination caused by the 5 to 7 per cent red cell ghosts present in the recovered cell suspension, the ghosts
present in the hypotonic supernatants were concentrated and checked for uptake of Cr⁵¹. The ghosts were washed twice in saline in a Sorvall centrifuge at 27,000 g for 10 minutes. Sixty per cent of the cells originally present in the incubation step were recovered as ghosts in this concentration. Three ml. of packed 100 per cent ghosts were washed an additional two times to free them of extracellular hemoglobin and were diluted 1:4 for testing. Microscopically, this washed ghost preparation consisted of <1 per cent erythrocytes, 40 per cent "black" ghosts (much attached hemoglobin), and 60 per cent "gray-white" ghosts (very little attached hemoglobin).

The ghost preparation was labeled with Cr⁵¹ under the usual conditions and assayed for radioactivity. The control sample consisted of 1 ml. of normal cells from the same original cell population treated in an identical manner. The...
radioactivity incorporated in the ghost preparation was 4.89 per cent of the control cell preparation. Since about 5 per cent of the isolated young cells in these experiments were ghosts, it may be expected that ghosts would comprise no more than 0.24 per cent of the radioactivity of the inagglutinable young cell preparation. This is an insignificant error in the determination of the inagglutinable cell frequency.

The results of the young cell experiments show that inagglutinability is not correlated with cell age. Young cells have the same proportion of inagglutinable cells as do the general cell populations. Moreover, the similarity in inagglutinable cell frequency between the isolated young cells and the total cell population indicates that other cell age fractions must be indistinguishable also. We have concluded, therefore, that older cells, comprising the bulk of the red cell population, do not contribute to the ICF by loss of antigenic sites, and, in fact, that cell age has no effect on the inagglutinable cell frequency.

DISCUSSION

Erythrocytes which fail to agglutinate in specific antiserum have been studied to determine the basis for their origin. Nongenetic origins—for example,
Table 2.—Response of Reirradiated Subjects

<table>
<thead>
<tr>
<th>Bird*</th>
<th>Inagglutinable Cell Frequency</th>
<th>Factor Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before</td>
<td>2 mo.</td>
</tr>
<tr>
<td>4293</td>
<td>4.12 x 10⁻⁸</td>
<td>4.58 x 10⁻⁸</td>
</tr>
<tr>
<td>89</td>
<td>2.37 x 10⁻⁴</td>
<td>1.31 x 10⁻⁴</td>
</tr>
<tr>
<td>948</td>
<td>7.10 x 10⁻⁵</td>
<td>9.50 x 10⁻⁴</td>
</tr>
<tr>
<td>averages</td>
<td>-0.21</td>
<td>-2.26</td>
</tr>
</tbody>
</table>

*All 3 were given a second dose of 360 r at 2.1 months.

mechanical removal of the antigenic sites, cells completely coated with antibody, or the transitory absence of the antigen—do not stand up under the weight of evidence against them and have been discussed in other reports.1,8 Indirect evidence against transitory loss of antigen is gleaned from control birds in the foregoing experiments, whose ICF remained constant over a long period of time, and from the results of the young cell experiments.

Peculiarities of the plant agglutinin—for example, presence of blocking antibodies and the low specificity of cross-reacting antibodies in general—likewise are not compatible with the type of data obtained in these experiments or with the accuracy of ICF determinations. Furthermore, the inagglutinatable cell frequency of a sample of blood is found to be the same regardless of the agglutinin used: specific immune anti-A human serum, absorbed, partially-purified P. lunatus lectin, or crude P. lunatus lectin.

Vascular anastomoses during fetal life can result in permanent erythrocyte antigen mosaicism,15 but this does not occur, of course, in oviparous animals such as pigeons. Polyspermy also results in natural chimeras and has been observed in pigeons.16 This type of chimera must be excluded from present consideration, however, since our birds are mated two to a cage. Other types of chimeras—for example, parabiotic or transplantation chimeras—also are not involved here but can give a significant percentage of "inagglutinatable" cells because of admixture of fetal tissue. Such a chimera might have both type A and type O blood, for instance, but the ("O") cells which remain free in anti-A serum are "inagglutinatable" in this restricted sense only.

Genetic origins of "A"-inagglutinatable red cells, especially mutation, are more complicated but more feasible in the light of the experimental results gathered so far. Mutation in the broad sense covers a variety of phenomena, notably point mutation, deletion, chromosomal aberrations, and nondisjunction. Several of these events have been reported following x-ray irradiation.

The variation in response to the different levels of x-ray used in these experiments proved to be a difficulty in interpreting the data. Some birds responded with an increased ICF as anticipated, but others failed to show any change or else responded negatively. The lower x-ray doses, especially 360 r, gave a better response than did high doses, and hence the total dose of 360 r was chosen as the optimum dose for subsequent experiments. These results are similar to those of Scheinberg and Reckel, who reported a maximum-response dose of 500 r for increasing the ICF in pigeons and who also found that high doses of x-ray, around 1000 r, failed to elicit any response.15 The relation of these results to selection is discussed in the companion paper.
Detailed data on the inheritance of the A2 agglutinogen of pigeons is currently being assembled, as is information showing the embryological development of the antigen. It is hoped that by using these data in conjunction with metabolic inhibitors the site of cellular injury following irradiation can be elucidated.

**SUMMARY AND CONCLUSIONS**

Experiments were carried out on the nature of "A"-inagglutinable cells. The effect of x-rays on the inagglutinable cell frequency was determined in pigeons by comparing the frequency before and after irradiation. Initial results in the treated birds were highly variable. Considerable reduction in the variability of response was obtained by control of dose, agglutinogen titer, breed, age and sex. Data was also obtained showing that there were individual differences in response to irradiation.

Significant increases in the frequency of the inagglutinable cells over that of the controls were obtained for both males amid females at 2 and 4 months following irradiation with 360 r.

The relation of cell age to inagglutinability was tested by comparing the inagglutinable cell frequency of isolated young cells with that of the total cell population. The comparable frequencies which were observed indicate that cell age is not related to "A"-inagglutinability.

**SUMMARIO IN INTERLINGUA**

Esseva effectuate experimentos relative al natura del cellulas inagglutinabile A. Le efecto de radios X super le frequentia de cellulas inagglutinabile esseva determinate in columbas per comparar le valores ante e post le irradiation. Le resultatos initial in le tractate aves
eseva altamente variate. Un considerabile reduction del area de distribution del responsas eseva obtenite per regular le dosage, le titro de agglutinogno, le linea genetic, le etate, e le sexo del aves. Eseva etiam obtenite datos demonstrante differentias individual in le responsa al irradiation.

Augmentos signifcative in le frequencia del inagglutinabile celularis in comparation con le valores de controlo eseva obtenite in aves de ambe sexos dua e quatro menses post le irradiation a un nivello de dosage de 360 r.

Le relation inter etate e inagglutinabilitate cellular eseva testate per comparar le frequencia deiagglutinabilitate inter isolate celular de juvne etates con illo in le population total. Comparabile frequentias eseva obtenite, indicante que le etate cellular non es relateionte con inagglutinabilitate A.

ACKNOWLEDGMENTS

Grateful acknowledgement is extended to Cmdr. Bodenlos of the Naval Medical Research Institute, Bethesda, Maryland, for use of the x-ray facilities there, and to Col. McChord and Mrs. Reeder of the Walter Reed Army hospital Blood Bank for drawing blood from our A1 donors. The authors also appreciate the suggestions made by Prof. M. R. Irwin and Dr. R. R. Babson in their review of this manuscript.

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