RADIOIMMUNE ASSAY FOR THE Xg(a) SURFACE ANTIGEN AT THE INDIVIDUAL RED CELL LEVEL

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Summary: An indirect radioimmune assay has been successfully used for the detection of the Xg(a) antigen on the surface of individual human red blood cells. The assay requires only microvolumes of reagents, is highly sensitive, and is equally reliable with fresh blood samples and with red blood cells stored in liquid nitrogen. The method has been used to estimate the number of Xg(a) binding sites. It may be useful to screen for quantitative isoalleles, as well as to investigate the alleged lack of participation of the Xg-locus in the inactivation of the X-chromosome.

The Xg(a) surface antigen is coded for by a gene, Xg^a, on the X-chromosome of man (1); a silent allele of this gene, Xg, behaves as a recessive, so that only two phenotypes can be distinguished in either sex. Extensive pedigree and population studies in humans have confirmed sex linkage for this gene, but have failed to provide precise mapping data with respect to other markers of the human X-chromosome (2). A peculiarity of this antigen is that heterozygous individuals (Xg^a/Xg) seem to contain a homogeneous population of Xg (a+) red cells (3), which is the only tissue for which the presence of the Xg(a) antigen has been clearly established (4). It is not clear whether this absence of mosaicism in the red cells is due to inadequacies of the techniques currently used for Xg-typing, to structural and/or developmental properties of the Xg(a) antigen, or to a lack of involvement of the Xg locus in the phenomenon of X-chromosome inactivation (5). The technique described in

Abbreviations:

PBS: 0.1M NaCl,11 mM KH_2PO_4, 13 mM Na_2HPO_4, 0.4 mM $MgSO_4$, pH 7.0.

BSA: Bovine serum albumin

PBS-azide-BSA: 0.1 M NaCl, 11 mM KH_2PO_4, 13 mM Na_2HPO_4, 0.4 mM $MgSO_4$, pH 7.0, 0.02 % sodium azide, 2% bovine serum albumin.

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this report permits the identification of the Xg(a) antigen at the individual red cell level, and therefore will be of particular value in the clarification of the above points.

Materials and Methods

Blood samples: The bulk of the red cells analysed in this study had been stored in liquid nitrogen in the form of frozen micro droplets for as long as three years. These bloods had been typed for the Xg(a) antigen by an indirect Coombs agglutination test soon after collection in Sardinia as part of a population survey (6). Freshly drawn samples of known Xg(a+) and Xg(a-) red cells from normal donors were included as controls in each set of determinations.

Antisera: One anti-Xg(a) serum was the generous gift of Dr. P. Rubinstein of the New York Blood Center. A second anti-Xg(a) serum was kindly made available to us by Ms. J. Mann of the Pfizer Company, Diagnostics Division, Groton, Conn., and also by Dr. R. Sanger of the University College, London. This serum is from the original patient in whom the antibody was described (1). Both antisera were derived from polytransfused patients and were processed prior to use according to standard absorption techniques, to remove antibody contaminants against known red cell antigens which were absent in the patients. However, the possibility that undetermined antibodies may still be present in the processed anti-sera cannot be excluded.

Anti-human-IgG sera was supplied by Drs. F. Siegal and U. Hammerling as 50% $(NH_4)_2SO_4$ precipitates of sera from rabbits immunized with human immunoglobulins.

Iodination of rabbit anti-human-Ig: A modification of the procedure of Montelaro and Rueckert was used (7). The reaction mixture contained 500 µg of antibody , 7.5 nmoles of KI and 2 to 5 mCi of carrier-free $Na[^{125}I]$ in 1 ml. of 0.1M PO_4 buffer, pH 7.0. The reaction was catalysed by chloramine-t; three aliquots (10 µl each) of chloramine-t were added at 10 minute intervals. After a total time of 30 minutes at 0°C, the reaction mixture was transferred to a dialysis bag, and dialysed against several rapid changes of PBS (0.1M NaCl, 11mM NH_2PO_4, 13mM Na_2HPO_4, 0.4mM $MgSO_4$, pH 7.0) to remove unreacted $[^{125}I]$ and chloramine-t. This was followed by extensive dialysis for several days at 4°C against a total volume of about 10 liters. Essentially all counts remaining after this treatment was trichloroacetic acid precipitable; the specific activities of the preparations ranged from 10^6 to 10^7 dpm/µg. After dialysis, the anti-human IgG was adjusted to a final concentration of 250 µg/ml in PBS containing 1% bovine serum albumin (BSA).

Radio-immune typing of red cells: Packed red blood cells were washed three times in PBS containing 0.02% sodium azide, and were resuspended in the same buffer at about 10^9 cells/ml. A 20 µl aliquot of the suspended red cells was mixed with an equal volume of the anti-Xg(a) serum, and the mixture incubated with agitation for one hour at room temperature. After three washes in PBS-azide (1 ml each), the red cell pellet was mixed with 40 µl of $[^{125}I]$-anti-human IgG and again incubated for one hour, with shaking, at room temperature. The cells were washed 6 times with PBS-azide containing 2% bovine serum albumin. Red cells incubated with the iodinated anti-human IgG in the absence of the anti-Xg(a) serum were included as controls.

The washed, labelled red cells were resuspended in 0.5 ml of PBS-azide-BSA, and spread on slides; after air-drying, the slides were fixed with 2%

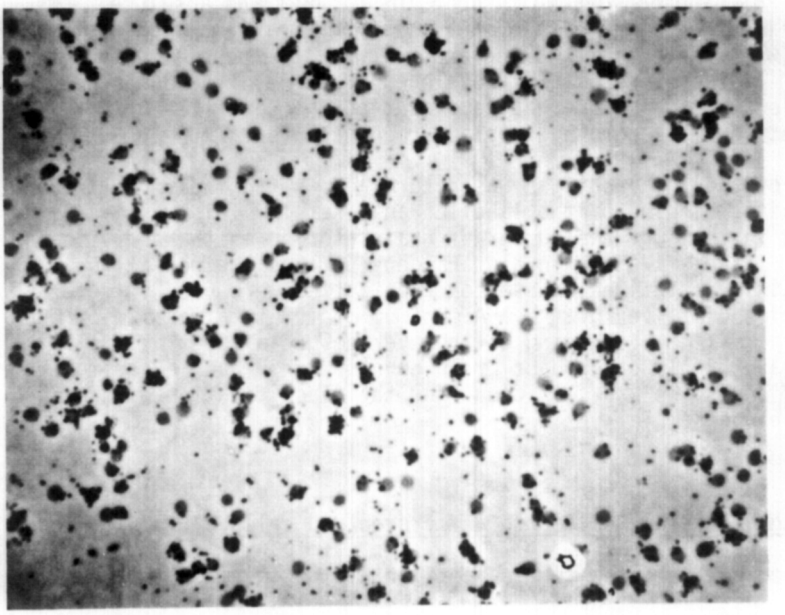
gluteraldehyde in PBS, and dehydrated through 70% ethanol and absolute ethanol. Dried slides were then dipped in autoradiographic emulsion (Kodak NTB, diluted 1:1 (w:v) with water), dried and allowed to expose at 4°C in light-tight boxes. Slides were usually developed at three different exposure times (the total length of exposure was dependent on the specific activity of the labelled antiserum) according to standard procedures.

The presence of the Xg(a) antigen was determined at the individual red cell level as specific binding of the labelled anti-serum. This was quantitatively expressed in terms of the number of grains formed on the individual red cells per day. Since fixation in gluteraldehyde makes the red cells impermeable to staining solutions, phase contrast optics were used to determine the distribution of silver grains. A minimum of 100 to a maximum of 600 cells were scored per slide, depending on the average grain count per cell. Duplicate slides gave highly concordant grain counts within each experiment, so that scoring of one slide was sufficient for the classification of the Xg-phenotype of each blood sample.

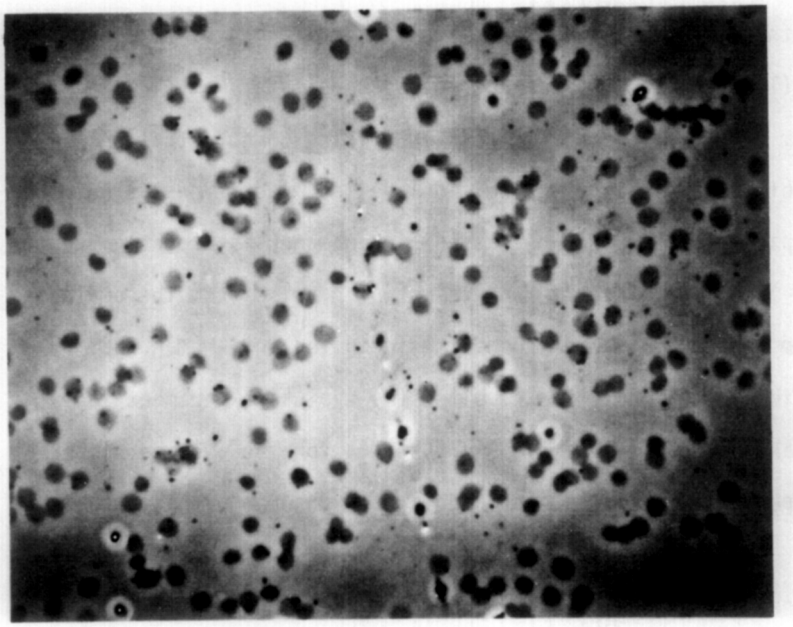
Results

1. Xg-typing of freshly collected and frozen red cells: Fig. 1 illustrates the detection of the Xg(a) surface antigen at the level of the individual red cells with our indirect radioimmuno-assay. The average number of silver grains on the Xg(a+) cells (upper photo) is strikingly higher than on the Xg(a-) cells (lower photo). Since both blood samples of Fig. 1 were freshly drawn from normal male donors, it is surprising to note that 5-10% of the red cells of the Xg(a+) samples were unlabelled. Additional studies to be reported separately, have shown that this phenomenon is probably due to cell aging. Xg(a-) cells show scattered grains which arise from the emulsion backgound and from non-specific binding of the iodinated anti-human IgG.

Fig. 2 describes graphically the results of a typical experiment in which six Xg(a+) and four Xg(a-) samples were scored for the average number of silver grains per cell after 1, 3 and 5 days of exposure to the photographic emulsion. In this particular experiment the discrimination between the positive and negative cells becomes unquestionable around day 3. However, additional experiments demonstrated that the exposure time required is strongly influenced by the titer of the anti-Xg(a) serum, and by the specific activity of the [^{125}I]-antihuman IgG. Unfortunately, the titer is influenced by the state of preservation of the antiserum and must be monitored regularly. The Xg(a+) red cell samples shown in Figure 2 include samples which had been



Xg (a+)
red cells



Xg (a–)
red cells

Figure 1
Autoradiographic identification of Xg(a) binding sites at the individual red cell level with the indirect radio-immune assay. The photos show the typical appearance of Xg(a+) and Xg(a-) red cells, both obtained from normal adult males of known genotype, after exposure to the anti-Xg(a) serum and to a rabbit anti-human Ig labeled with radioactive iodine. The pictures were taken after a five day exposure of the slides to the photographic emulsion. The few scattered grains seen in the bottom picture are present also when the first reagent is omitted and must therefore be attributed to background or to aspecific binding of the anti-human Ig serum.

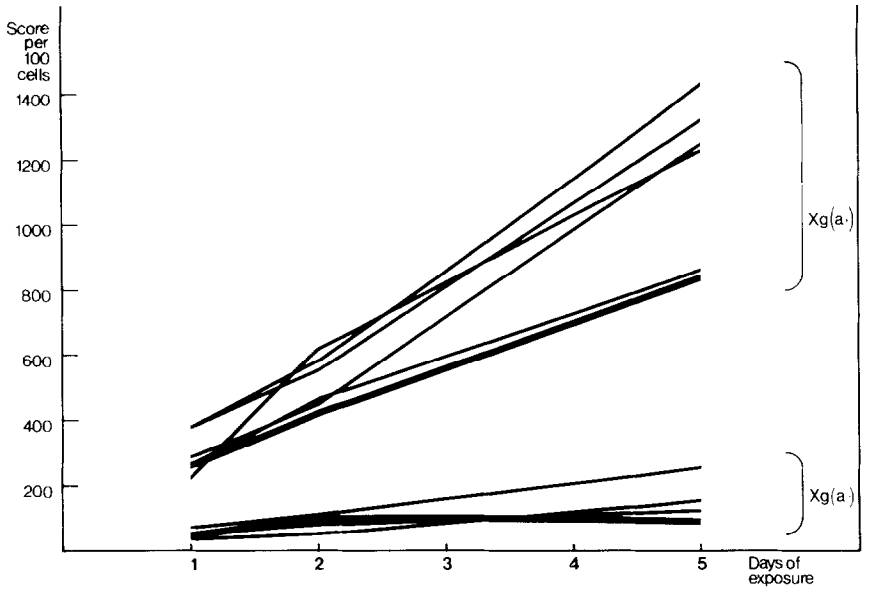


Figure 2

Rate of grain formation versus exposure time in six Xg(a+) and four Xg(a-) samples of red cells derived from normal adult males. The lines drawn in bold characters identify the only two freshly collected samples used in this experiment. All other samples were thawed from liquid nitrogen after storage for three years in the form of micro-droplets.

stored in liquid nitrogen; these samples show a higher average grain count per cell than do the fresh red cells. This finding may be the result of differences in mean cell age since, on thawing of frozen sample, there is some preferential hemolysis of the oldest red cells.

To date, 50 erythrocyte samples have been tested using the indirect radioimmunoassay. All 30 Xg (a+) bloods were correctly classified while two of the 22 Xg (a-) bloods were misclassified. The two aberrant samples, when incubated with iodinated antihuman IgG in the absence of anti-Xg(a) serum, exhibited the same high average of grain counts per cell as in the complete reaction. The Xg(a+) and Xg(a-) controls treated in the same manner had average grain counts as low as the Xg(a-) cells of the complete reaction. This finding led us to conclude that the two misclassified samples carry, on their red cell surfaces, autoantibodies that react directly with the labeled

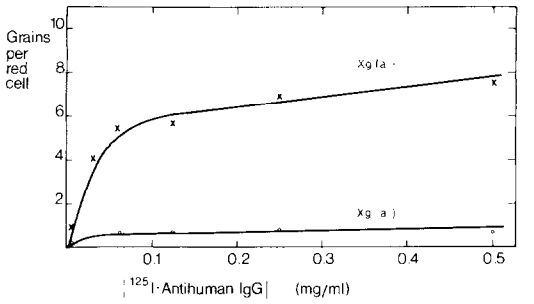


Figure 3

Saturation curve of Xg(a) binding sites on red cells of a normal adult male. There are about 2.5 grains formed per day per Xg(a+) red cell. This leads to a crude estimate of approximately 9000 binding sites per cell (see text).

anti-human IgG. When the anti-Xg(a) serum was absorbed with red cells from a misclassified individual, and then tested on positive red cells, the specific anti-Xg(a) activity was unaffected, thus providing further evidence of the absence of Xg(a) antigen in these red cells. This matter is the subject of further studies currently underway in this laboratory.

2. Estimate of Xg(a) binding sites on the red cell surface. A preliminary attempt has been made to calculate the number of binding sites available on the red blood cell surface for anti-Xg(a). The average number of grains per Xg(a+) red cell per day is 2.5 under conditions at or near saturation (Figure 3). Since each grain arises from about 5 disintegrations, there are approximately 10×10^{-3} dpm per minute per cell. The specific activity of the IgG was 3×10^{6} dpm per minute per μg, and the molecular weight is about 10^{5}; there are then 1.5×10^{-14} disintegrations per μ mole per cell. This figure, divided by the Avogadro number, yields an estimate of 9000 sites per cell. This finding must be confirmed by further studies of the same kind using purified preparations of anti-Xg(a) antibodies; however, it is reasonable to assume that the above estimate is of the correct order of magnitude.

Discussion

The indirect radio-immuneassay described in this report allows for accurate identification of the Xg(a) surface antigen. Major advantages of this procedure are:

(i) the presence of Xg(a) substance can be scored quantitatively and at the level of the individual erythrocyte;

(ii) the sensitivity of the method is several logs greater than that afforded by the conventional indirect Coombs agglutination test;

(iii) only a microamount of red cells is needed for the assay, such as can be obtained by finger-prick;

(iv) only 20 µl of an appropriate dilution (1:20-1:100) of the antiserum are needed for each sample (a far from trivial detail, in view of the shortage of the antisera and the extreme rarity of suitable donors);

(v) the efficiency and reliability of the test are essentially unimpaired when the Xg-typing is performed on frozen erythrocytes stored in liquid nitrogen for as long as 3 years.

It should be noted that this assay is somewhat more cumbersome and expensive that the conventional indirect agglutination test, which remains the method of choice for routine blood bank testing and population studies. However, in view of the above advantages it is expected that the new assay will be crucial in finalizing the issue of the existence of an X-inactivation-dependent mosaicism for Xg(a) in the red cells of heterozygous women, or of and X-independent dosage effect in the homozygotes for the normal allele (Xg^a/Xg^a). Studies bearing on these points are being conducted in our laboratory and will be the subject of a separate report.

As explained above, the detection of false positives with this new technique can be easily avoided with the use of appropriate controls or with the use of purified anti-Xg(a) IgG. In our experience, the problem of misclassification is particularly serious when typing nucleated cells. In the absence of purified antibodies, this difficulaty can be circumvented with an

indirect test such as preliminary absorption of the anti-Xg(a) serum with the nucleated cells to be typed, followed by testing the residual anti-Xg(a) activity on known Xg(a+) and Xg(a-) red cells with the above test. We have successfully employed this procedure to screen for the presence of the Xg(a) antigen on fibroblasts (unpublished observations).

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