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QUANTITATION OF ANTIBODY BINDING TO CELL SURFACE ANTIGENS BY X-RAY FLUORESCENCE SPECTROMETRY

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Summary

An X-ray spectrometric method has been developed to quantitate antibody binding to whole cell surfaces in order to obtain a distribution of binding within a population of cells. The method involves incubation of target cells with ferritin-labeled antibody. Analysis of prepared samples in a modified transmission electron microscope with an X-ray detector and data analysis equipment, yields quantitative results on the binding of labeled antibody to individual cells. The binding of anti-2,4-dinitrophenol serum to Chinese hamster ovary cells with attached 2,4-dinitrophenol haptens was measured by X-ray spectrometry. Measurements of attached hapten by a radioisotopic marker correlated with the X-ray spectrometric determination of bound antibody. The use of synchronized cells in metaphase and G1 phases of the cell cycle permitted investigations into the binding per unit surface area. The distribution of antibody binding among a given population of cells was related to the surface area of the cells.

Introduction

Quantitation of cell membrane antigens or receptors has been approached by various immunological methods including direct binding of radiolabeled antibody [1,3], indirect isotopic antiglobulin techniques [4,5] and paired-label alloantibody techniques [6,7]. These approaches allow calculation of a specific binding ratio relative to control antiserum and an estimate of the mean number of cell surface sites from the specific activity of the bound radiolabeled antibody, assuming a given molecular weight for immunoglobulin G (IgG), condi-

tions of antibody excess, and univalent antibody binding. An alternate method [9], utilizing the inhibition of erythrocyte lysis as an indicator, allows an estimate of the mean number of C1-fixing antibody molecules per cell.

These methods require large numbers of cells and the resulting data yield only an estimate of the mean for a population of cells. An important aspect that has not been considered is the distribution of the number of surface antigens per cell within a population. How variable can the number of sites be, between individual cells? Are the deviations from the mean large or small, or dependent on cell type? These questions can only be answered by a technique which examines the antigenic sites one cell at a time. The development of the fluorescent cell sorter [10] has permitted qualitative analysis of the distribution of antigen-expressive cells within a population, and, more importantly, their separation. Technological limitations have prevented the calibration of this fluorescent data for quantitation of antibodies bound per cell. However, the relative fluorescence intensity can be measured [11]. Similarly, photometric units per cell have been used to measure the binding of radiolabeled antibody by autoradiography [12] but radioactive reference sources have not been developed for absolute determinations.

In our laboratory, we have been involved in quantitative studies of antigens on human tumor cells, and have developed a single cell technique using some previously established procedures [13,14]. The objective of the studies reported in this paper was to quantitate this technique and to confirm the accuracy of the method.

Materials and Methods

The basis for the quantitative method was the coupling of the antibody to a label, and quantitation of the label by means of X-ray spectrometry. The demonstration that the electron microprobe system was truly quantitative necessitated a detailed study with some means of correlating numbers of bound antibodies to numbers of available antigens. To avoid possible problems in identifying non-specific binding, a highly specific system was also needed. Therefore, rabbit anti-2,4-dinitrophenyl-bovine serum albumin was chosen as the specific antibody, and 2,4-dinitrophenol as the hapten in the test system.

In order to confirm the accuracy of the system, it was necessary to quantitate the attachment of the hapten to the cell surface and correlate these results with the X-ray spectrometric quantitation of bound labeled antibodies. Also, to demonstrate that the attachment of hapten and subsequent antibody binding was truly related, data was accumulated in a range where there were excessive haptens for corresponding antibody binding (due to steric hindrance). At these "saturation" values, the maximum number of antibodies were bound to the cell. To verify this fact, synchronized cells were used, allowing quantitation at two different cell sizes.

Rabbit-anti-2,4-dinitrophenyl-bovine serum albumin was obtained from Miles-Yeda (Israel). Six times crystallized ferritin was obtained from Polysciences, (Warrington, IL). The antibody was coupled to ferritin using the procedure of Singer and Schick [15] using toluene 2,4-diisothiocyanate as the coupling agent. A sucrose density gradient purification procedure [16] has

been developed and used in our laboratory to eliminate free antibody and avoid aggregation problems. The band of ferritin-antibody and uncoupled ferritin was recovered from the gradient and stored at 4°C.

The synchronized cells were obtained by a modification of the procedure o of Stubblefield and Klevecz [17]. A Colcemid block was administered to the cultured Chinese hamster ovary (CHO) cells for 2 h. A 5 min agitation of the culture flask was used to harvest mitotic cells, which were then poured off with the medium, centrifuged and resuspended in cold medium. The cell concentration was determined via hemacytometer and the mitotic index obtained by an aceto-orecin stained squash preparation. This index was always greater than 95%. For data requiring metaphase cells, the cells were continually maintained in suspension with Colcemid to prevent progression into the G1 phase. For experiments requiring G1 phase cells, the metaphase cells were resuspended in cold medium (Colcemid), plated into a small tissue culture dish, and incubated at 37°C for 1 h. After this period, the cells were washed with saline, removed from the dish with trypsin, and suspended in Hanks balanced salt solution. These G1 phase cells were then ready for antigen attachment or use as controls. For antigen attachment, aliquots of each type of cell (metaphase and G1 phase) were taken for size determinations in a Coulter counter and Channelyzer.

The attachment of antigen was based on the procedures of Rittenburg and Pratt [18] as modified by Scornick [19]. The sulfonic acid derivative (2,4,6-trinitrobenzenesulfonic acid) is more reactive [20], but only 2,4-dinitrophenol was readily available in radiolabeled form; 2,4-dinitro[4-^{14}C]phenol was obtained from New England Nuclear (Boston). A working stock solution of $3.43 \cdot 10^{-5}$ Ci/l labeled 2,4-dinitrophenol dissolved in 0.28 M cacodylate buffer (pH 6.9) was prepared. This solution was prepared at the given concentration, because of preliminary studies with 2,4,6-trinitrobenzenesulfonic acid which yielded an estimation for the maximum binding of hapten to the cell surface. Varying amounts of the stock solution and varying numbers of cells were used to facilitate a wide variation of antigen attachment.

The plasma membrane localization of dinitrophenol attachment was independently confirmed by immunofluorescence microscopy of viable unfixed cells. Even under conditions where methanol fixation of hapten-tagged cells allowed distinct nuclear staining by an anti-DNA antibody, (from serum from a patient with lupus erythematosis), only a halo-type membrane fluorescence was produced by a rabbit anti-dinitrophenol antibody, using the appropriate species-specific, fluorescein-conjugated, anti-IgG reagents. In no case was capping observed.

After an incubation of the cells with hapten at room temperature (22°C) for 15 min, the cells were washed with the appropriate buffers (modified barbital buffer and modified barbital buffer with glycine) [19]. The cells were then washed with Hanks solution and resuspended in 1 ml of labeled antibody solution. Generally, $1-3 \cdot 10^6$ cells were reacted with the ferritin-labeled anti-dinitrophenol antibody. The cell suspension was incubated for 1 h at 37°C and then washed twice with Hanks solution. After the final wash, an aliquot of cells was ready for spinning onto electron microscope grids in an apparatus designed especially for this type of sample preparation. After a 10 min centrifugation, these cells were fixed with glutaraldehyde and then air dried. Another aliquot

of the cells was added to a vial with 10 ml dioxane-base liquid-scintillation fluor. These vials with fluor had been precounted over 1000 min to obtain an accurate background (relative standard deviation less than 3%). The vials were then counted again after the cells were added, for over 1000 min due to the relatively low activity.

X-ray spectrometric analysis of all samples was accomplished in a modified Hitachi HU-10A transmission electron microscope. A sample under bombardment by an electron beam produced a spectrum of X-rays. The number of characteristic X-rays is proportional to the number of atoms under bombardment [21]. Therefore, for a 1 : 1 ferritin/abtibody ratio (confirmed by analytical ultracentrifugation) and the knowledge of the iron content in ferritin, the number of bound antigenic sites can be determined by measuring the intensity of the iron K_α X-ray line. This spectrum is detected by some type of energy dispersive detector. Requirements for the detector are that it must detect low energy X-rays and have high energy resolution to improve the signal-to-noise ratio.

These requirements make the preferred choice lithium-drifted silicon (Si(Li)) detectors with thin beryllium windows. Si(Li) detectors are highly efficient over the energy range from 2 to 20 keV (including iron K_αX-rays at 6.4 keV).

The arrangement of sample holder, detector and collimator, in relation to the beam is shown in Fig. 1. It should be noted that this arrangement permitted the use of transmission optics to localize the sample, and, therefore, analyze one cell at a time. The accelerating voltage was 50 kV, magnification was 1000 X. Current densities, measured in a Faraday cup, were usually between 1 and 15 nA/cm^2.

Calculation of iron atoms detected was based on the following relationship:

$$M = \frac{KGFZ^2N}{AI}$$

where M = mass per unit area (g/cm^2); A = Avogadros number ($6.02 \cdot 10^{23}$ atoms/mol); I = current density (A/cm^2); K = atoms constant (atoms-amperes-

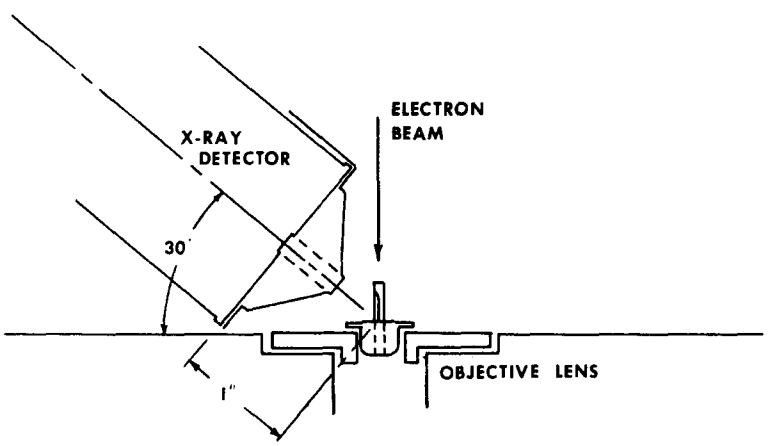

Fig. 1. Cross-sectional view of sample holder, detector, and collimator location in relation to electron beam.

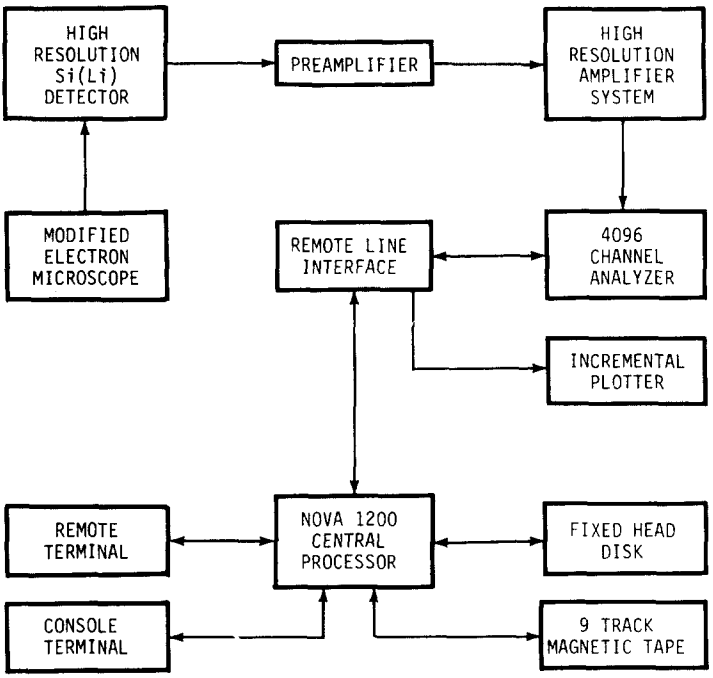

Fig. 2. Schematic of electron microprobe system with associated data analysis equipment.

seconds/cm^2); G = atomic weight (g/mol); F = intensity correction factor, including K fluorescent yield, sample absorption and window absorption; Z = atomic number; N = counts in peak (cps). Using thin copper, nickel and iron foils on known thickness (mg/cm^2), the constant K was calculated. For the iron

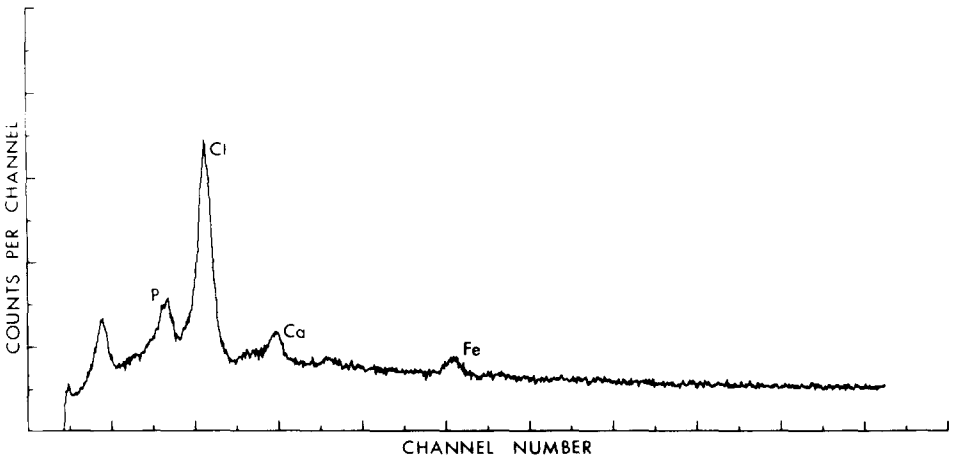

Fig. 3. X-ray energy spectrum from a single Chinese hamster ovary cell. The iron peak (K_αX-ray line) was from iron in ferritin, the P, Cl and Ca peaks were from other cellular ions; and the gently slopimg background was due to Bremsstrahlung. Major divisions on the abscissa are every 100 channels, energy calibration was 12.7 eV/channel.

calculation, most of the constants were combined, and using units of convenience:

$$T = (KA)\frac{N}{I}$$

where T = number of iron atoms detected; KA = modified atoms constant ($7.494 \cdot 10^8$ atoms nA/cm^2); N = number of cps in peak); I = current density (nA/cm^2). The data was processed by the instrumentation system represented in Fig. 2. A spectrum was acquired in the multichannel analyzer and data reduction was performed by the instrumentation shown. Quantitative calculations were possible with just the multichannel analyzer, but the system shown allowed rapid, more complete on-line data analysis. An example X-ray spectrum is shown in Fig. 3.

Results

The average number of hapten molecules per cell was calculated using radioactive measurement data (corrected for background and counter efficiency), and known specific activity. For the X-ray spectrometric analysis, 50 cells were counted (5 min count for each cell) in each of the 12 samples. The numbers of bound antibodies were calculated by dividing the number of iron atoms detected by X-ray spectrometry, by 2000, the average number of iron atoms per ferritin molecule [22].

This data was then correlated with the radioactive measurement data from liquid scintillation counts, to yield curves for metaphase and G1 phase, of the comparison of bound antibodies to attached haptens. The curves are shown in

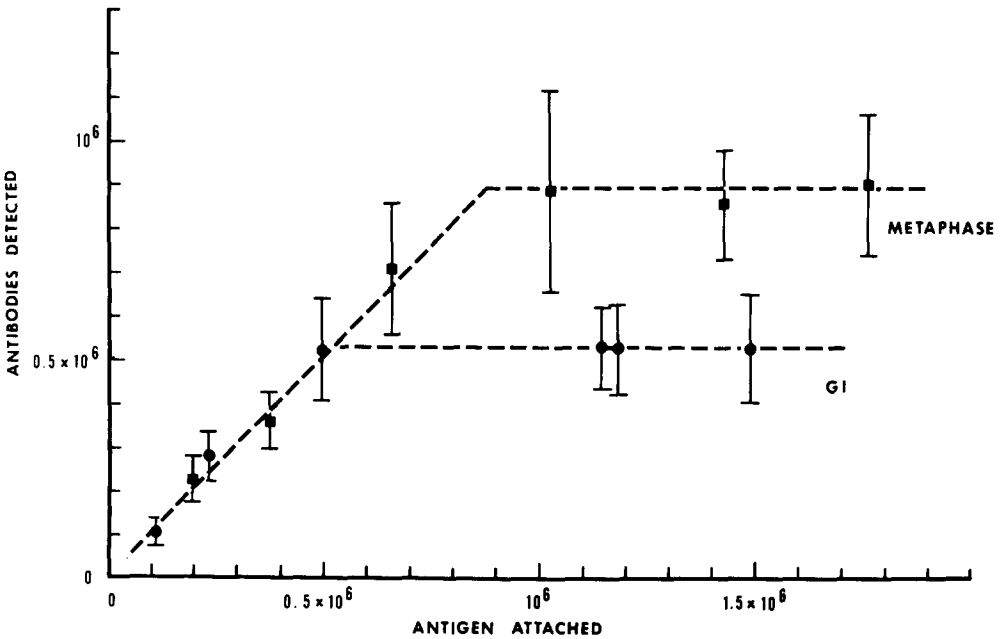

Fig. 4. Saturation curve comparing bound labeled antibody as a function of attached antigen.

TABLE I

SIZE AND VARIABILITY OF SYNCHRONIZED CHINESE HAMSTER OVARY CELLS AS DETERMINED BY COULTER COUNTER

	Metaphase	G1
Volume	1900 ± 280 μm^3	1100 ± 200 μm^3
Radius	7.7 ± 1.5 μm	6.5 ± 1.2 μm
Surface area	750 ± 110 μm^2	530 ± 100 μm^2

Fig. 4, where the vertical bars are the standard deviations for the 50 cells counted at each point. It should be noted that these vertical bars are measures of variability and are not system errors. The X-ray data presented has also had a value of $0.0517 \cdot 10^9$ iron atoms/cell subtracted. The above value was reached by analysis of 40 CHO cells (no hapten) which had been incubated with the labeled antiserum and previous procedures followed. The $0.0517 \cdot 10^9$ iron atoms therefore represent system backgrounds, endogenous iron, as well as non-specific attachment of labeled antibody or ferritin to the cell surface. Another control series of 40 CHO cells which had been incubated with normal rabbit serum yielded a value of $0.0414 \cdot 10^9$ iron atoms per cell.

The data on cell size was determined by analysis in a Coulter counter and Channelyzer. Aliquots of the CHO cell suspension (cells in either metaphase or G1 phase) were compared with polystyrene calibration beads of known volume. From the cell volume distribution, points at one standard deviation from the mean were determined. This was fone by a graphic integration of the area under the peak, and trial and error determination of the deviations. The cell radius and surface area was then calculated at the three points of the distribution (mean and one standard deviation above and below the mean) (Table I). The relative standard deviation for cell surface areas varied from 15 to 20% between experiments for both phases of the cell cycle.

Discussion

We have adapted the principles of X-ray fluorescence spectrometry for the detection and quantitation of antibodies bound to cell surface components. These studies were conducted to determine the accuracy and limits of detection for this system, whereby an immunological probe can be used to quantitate specific determinants expressed on the cell membranes.

The curves shown in Fig. 4 depict maximum limits of accurate quantitation for this system. For the larger metaphase cells, the number of antibodies bound approaches 900 000. The asymptote is a result of a situation where there are so many haptens in such close proximity that antibodies cannot bind to every hapten, due to steric hindrance. For the smaller G1 phase cells, the asymptote is 530 000 bound antibodies. These numbers represent maximum limits of accurate quantitation for the 2,4-dinitrophenol study, and yield an estimation for maximum accurate quantitation for other antibody studies. The reason that that is only an estimation is due to the distribution of antigen over the cell surface. The 2,4-dinitrophenol molecule binds preferentially to certain amino acid

residues of proteins [20]. Assuming the current models of the cell membrane are correct, with protein units in a lipid 'sea', then the haptens should be localized in high density regions on the cell surface. Naturally occurring antigens may be more uniformly distributed, allowing a higher limit for accurate quantitation. Much higher binding (millions of antibodies per cell) has been reported for horse antibody binding to HeLa cells [7].

At the saturation levels of antibody binding, an average for the number of antibodies per unit cell surface area can be calculated. Using the Coulter counter data of 750 and 530 μm^2 for surface area of metaphase and G1 phase cells, respectively, the resulting averages are 1200 and 1000 antibodies/μm^2. These values are within ranges for maximum horse antibody binding to HeLa cells [7].

Another calculation can be made to correlate the binding per unit surface area with actual physical measurements of cell size. The areas of the two different phases of synchronized cells (from Coulter counter data) can be divided to yield a ratio of 1.4 (metaphase area divided by G1 phase area). A ratio of the maximum antibody binding (900 000 for metaphase cells divided by 530 000 for G1 phase cells) can be calculated also, yielding 1.69. A theoretical value of 1.58 can be obtained by calculating the ratio of surface areas, assuming one spherical cell divided into two equal spherical cells each with exactly half the volume of the original cell. The physical measurement ratio of 1.4 is not statistically different from the ratio of 1.69 from saturation antibody binding values. It should be stressed that these values are statistically different from a ratio 1.0, verifying that maximum binding is a function of cell surface area. In addition, the difference between the 900 000 bound antibodies for metaphase cells and 530 000 for G1 phase cells is significant at the 95% confidence level.

Another important parameter is the lowest number of bound antibodies that can be detected. This value is usually determined by the statistical significance of the iron X-ray line above Bremsstrahlung background which is proportional to the mass of the sample. Therefore, for large cells, the minimum detectable limit is higher than for small cells. The CHO cells, being larger than most cells we have analyzed, had lower limits of 30 000—50 000 antibodies. Smaller cells, for example murine fetal liver cells, have lower limits near 10 000 simply due to their smaller mass. Cell lines with higher values of endogenous iron would have a correspondingly higher minimum detectable limit. Instrumentation modifications are expected to improve these lower limits by a factor of 2.

A strong point in the discussion of absolute quantitation of bound antibodies, is demonstrated in the curves in Fig. 4. At levels below saturation binding, the ratio of bound antibodies to attached haptens is 1, for the range of 100 000—700 000 for metaphase cells and 100 000—500 000 for G1 phase cells. This linearity in the curves supports the calibration for absolute iron quantitation for this system.

One of the primary advantages of this system over the quantitative techniques is that it determines bound labeled antibodies one cell at a time and a very small sample size can be used. When a number of cells from one sample are analyzed, a distribution of the antibody binding among the population of cells is determined. For the study with 2,4-dinitrophenol as an artificially attached hapten, the distribution should be consistent with the cell size distri-

bution. As seen in Table I, the relative standard deviations for cell surface areas were approx. 15 and 19% for the metaphase and G1 phase cells, respectively. The corresponding values from X-ray spectrometric data were always within the range 15—24%. This, again, reinforces the view that this method can yield quantitative information about the distribution of bound antibodies within a cell population.

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