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Expression of Major Histocompatibility Complex Antigens on Macrophages: Correlative Study Using Flow Cytometry, Radioimmunoassay, and Colloidal Gold Immunolabeling

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EXPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX ANTIGENS ON MACROPHAGES: CORRELATIVE STUDY USING FLOW CYTOMETRY, RADIOIMMUNOASSAY, AND COLLOIDAL GOLD IMMUNOLABELING

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Abstract

Correlative scanning electron microscopy (SEM), radioimmunoassay (RIA), and flow cytometric analysis were used to characterize levels of class I and class II major histocompatibility complex-encoded (MHC) antigen expression on peritoneal exudate cells of mice chronically infected with Chlamydia psittaci. Analysis of peritoneal macrophages by all three techniques revealed a marked induction of H-2 K,D (class I) and I-A, I-E (class II) antigens on cells from infected C3H mice when compared to uninfected controls.

Scanning electron micrographs further document that the increases in class I and II MHC antigens are due to an increase in Ia/H-2 bearing cells as well as an increase in MHC molecules/cell. These immune macrophages have a flattened morphology, almost completely devoid of the membrane ruffles and villi which are characteristic of control peritoneal macrophages.

These studies suggest that while both flow cytometry and RIA can provide an accurate quantitative estimate of antigen expression in a cell population, the immunogold labeling technique can allow visualization of individual cells and additional analysis of the topographical distribution of cell surface antigens.

Key Words: Macrophage, Major Histocompatibility Antigens, Scanning Electron Microscopy, Colloidal gold, Flow cytometry, Radioimmunoassay.

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Introduction

Macrophages play an important role in the activation of T lymphocytes. This activation requires recognition by the T cell of both histocompatibility antigens and foreign antigens on the macrophage cell surface. Although it is clear that this co-recognition of histocompatibility antigens and processed foreign antigen by the T cell leads to activation, the spatial relationship of these components on the macrophage cell surface in situ still is not well understood (Cowing, et al., 1978). In this study, we examine the effects of in vivo administration of a bacterial antigen, Chlamydia psittaci, on macrophage morphology and histocompatibility antigen expression, using the techniques of scanning electron microscopy (SEM), radioimmunoassay (RIA), and flow cytofluorometric analysis. Macrophage response to this organism is of interest both because Chlamydia causes trachoma and sexually transmitted disease and because macrophages serve as antigen-presenting cells for T cell activation (Schacter and Caldwell, 1980).

By the three techniques used, a marked increase was observed in the relative percentage of Ia-positive macrophages in the peritoneal cavity of C3H mice which were immunized and subsequently challenged with Chlamydia. In addition, SEM studies revealed that the macrophages obtained from immune mice show significant heterogeneity with respect to the expression of Ia (class II) antigens and that in this system high levels of Ia antigens are found on one macrophage morphological subpopulation present as flattened cells lacking surface ruffles and villi.

Materials and Methods

Animals

Adult male C3H mice were purchased from Sprague-Dawley Co., Indianapolis, Ind. and were certified virus-free. The MHC haplotype of this strain is H-2K^k, D^k, I-A^k, I-E^k.

Induction and Isolation of Inflammatory Macrophages

Inflammatory macrophages were elicited by intraperitoneal injection of 1 ml of 3% thioglycollate broth (TG, Difco Laboratories, Detroit, MI) 5 days prior to harvest. Peritoneal cells were harvested from C3H mice by peritoneal lavage and cultured at $2x10^6$ cells/ml in RPMI 1640 media (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Inc. Logan, UT). Macrophages were purified by adherence of the

cells at 37°C for 2 hr in a humidified atmosphere of 7% CO_2, after which nonadherent cells were removed by gentle but thorough washing.

Induction and Isolation of Chlamydia-sensitized macrophages

Mice were immunized by subcutaneous injection of $5x10^2$ viable Chlamydia psittaci elementary bodies, or mock-immunized by a similar injection of PBS, as previously described (Byrne and Faubion, 1982). Immune mice were challenged intraperitoneally 10 days later with 10^6 viable Chlamydia organisms and immune macrophages obtained by peritoneal lavage 5 days after challenge. Control exudates were elicited by TG in mock (PBS)-immunized animals on the same time schedule.

Monoclonal Antibodies

Hybridoma cell lines used as the source of monoclonal antibodies were obtained from the American Type Culture Collection (Rockville, MD) and included the following: 10-2.16 (Anti-I-A^k (Oi, et al., 1978)); 14-4-4 (Anti-I-E^k (Ozato et al., 1980)) MK-D6 (Anti-I-A^d (Kappler et al., 1981)); and 16-1-2 (Anti-H-$2K^kD^k$ (Ozato et al., 1980)). Cell lines were cultured in RPMI 1640 medium supplemented as described above. Monoclonal antibodies were purified from culture supernatants by protein A affinity chromatography.

Flow Cytometry Analysis

For cytofluorometric analysis, 10^6 washed peritoneal exudate cells were stained directly with fluorescein isothiocyanate-conjugated monoclonal antibodies by incubation for 15-30 minutes on ice. Following this incubation period, the cells were washed, resuspended in approximately 0.5 ml RPMI with 5% serum and 0.02% sodium azide, and analyzed on an Epics-C flow cytometer (Coulter, Inc. Hialeah, FL).

Protein A-gold Complex Formation

Colloidal gold granules having average diameter of 24 nm, Au_{24}, were prepared by reducing $HAuCl_4$ with trisodium citrate (Frens, 1973). The minimum amount of Staph protein A (Sigma, St. Louis, MO) necessary to stabilize the colloidal gold was then determined from an adsorption isotherm to be 12 µg protein A/ml gold solution.

Ten ml of gold solution (pH 7.15) was added to a 10% excess of protein A with gentle stirring. After 5 minutes, 0.5 ml of freshly prepared and prefiltered (Millipore 0.45 µm) 1% polyethylene glycol (MW. 20,000) was added to prevent aggregation. The protein A labeled gold (pA-Au) was centrifuged in polycarbonate tubes in an angle rotor at 10,000 rpm for 30 minutes. The supernatant was discarded and the concentrated red pool was resuspended to 2 ml with 0.2 µ filtered, 0.1M HEPES.

Electron Microscopy

All samples were fixed in 2% glutaraldehyde in 0.1M HEPES for 1 hr at 22°C. Washed specimens were then incubated in 0.2M glycine for 30 minutes to block free aldehyde groups. Surface histocompatibility antigens were labeled indirectly with the appropriate monoclonal antibody (20 min., 22°C) followed by Au_{24}-protein A (20 min., 22°C). The cell membranes were then stabilized with 5% glycerol in buffer for 5 minutes before dehydration through a graded series of alcohols to absolute ethanol. Samples were dried by the critical point method utilizing molecular sieve dried CO_2 as the transitional fluid; they were then sputter coated with 3-5 nm of gold and examined on a JEOL JSM 35C scanning electron microscope (SEM) at 25 kV accelerating voltage.

Radioimmunoassay of Macrophages

Macrophages were plated into 96-well microtiter plates at a concentration of $2x10^5$ cells in complete medium and allowed to adhere for 1 hr at 37°C/7% CO_2. Nonadherent cells were then removed by repeated, thorough washing of the monolayer with PBS. The remaining adherent cells were fixed in 1% paraformaldehyde and stored at 4°C until assay.

For the cell binding assay, 25 µl of monoclonal antibody (250 µg protein/ml) was added to triplicate wells and incubated for 1 hr at 22°C. Following 2-3 washes with PBS, 50 µl of ^{125}I-Staph protein A were added to the cells such that 50 µl ^{125}I-protein A corresponded to approximately 80,000-100,000 cpm. The ^{125}I-protein A was allowed to incubate with the macrophages for 1 hr at 22°C. Cells were again washed and the wells were separated and counted in a gamma counter. Iodination of the protein A was performed according to the iodogen method (Fraker and Speck, 1978). Standard controls for the assay always included cells incubated with ^{125}I-protein A both alone and following incubation of the cells with a control monoclonal antibody. The monoclonal antibody MK-D6 (anti-I-A^d) was used in RIA, flow cytometric and scanning electron microscopic analyses as the control for non-specific antibody binding. This antibody does not react with the I-A antigens expressed on macrophages from mice of the H-2^k haplotype. Cell number was quantitated by DNA measurement using a Hoechst dye (Labarca and Paigen, 1980) and antibody binding subsequently expressed as cpm bound/10^5 cells.

Results

Morphological Characterization of Adherent Exudate Cells

Initial SEM observations were made on adherent cells elicited by thioglycollate injection or by the in vivo administration of Chlamydia.

Scanning electron micrographs reveal that both the control macrophages and macrophages from infected mice are comprised of several morphological subtypes (Fig. 1). These include rounded cells with dense surface ruffles, (Fig. 1, arrow A), and flat, well-spread macrophages (Fig. 1 arrow B). Although all morphological subtypes are observed in both preparations, the control and immune populations differ in the relative number of cells in each morphological subpopulation. The most common morphology in the immune macrophage population is the extensively spread cell with few membrane ruffles or microvilli.

MHC Antigen Levels

Levels of MHC antigen expression were then evaluated by RIA and flow cytometry on both control and immune cell populations. Analysis of antigen expression by RIA revealed that macrophages from immune mice have levels of surface I-A antigens which are 4.9-fold higher than similarly labeled cells from mock-immunized mice (Table 1, 4747 cpm bound/10^5 cells as compared to 962 cpm). Immune macrophages also showed a 4.2-fold increase in the levels of expression of I-E antigens compared to control cells; H-2 antigens were 1.9-fold higher in these immune cells. In each case, the levels of non-specific labeling with control antibody MK-D6 are indicated in the first column. Thus, analysis with this technique revealed differences in the total amount of antigen expressed on

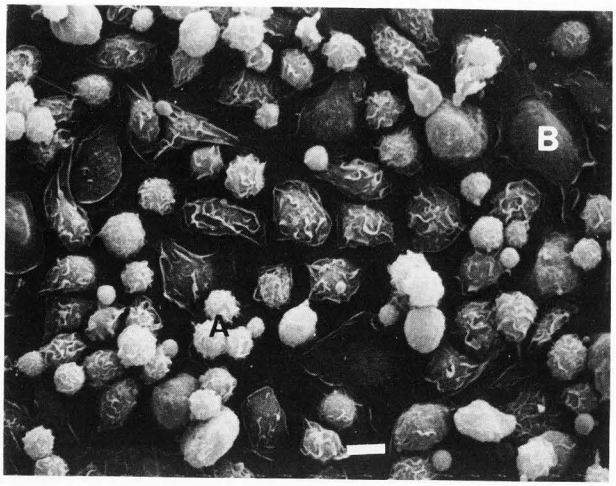

Figure 1. Characterization of the peritoneal exudate population elicited by in vivo administration of Chlamydia psittaci. Two basic morphological subtypes are seen: extensively ruffled rounded macrophages (A) and uniformly spread cells lacking membrane ruffles or villi (B). Bar = 10 μm.

TABLE 1. Comparison of I-A, I-E, and H-2K,D molecules on mock-immunized and Chlamydia-sensitized macrophages

Monoclonal Antibody[a]		Amount of Expressed Antigen (cpm ± SEM)[b]	
Specificity	Cell Line	Mock-immunized	Immune
Anti-I-A^k	10-2.16	962 ± 16.9	4747 ± 171
Anti-I-E^k	14-4-4	971 ± 50.0	4148 ± 163
Anti-H-$2K^k$,D^k	16-1-2	667 ± 2.83	1269 ± 89.1
Anti-I-A^d	MK-D6	895 ± 25.7	1095 ± 53.1

[a]Monoclonal antibodies from the indicated cell lines were used as the first antibody in the RIA (see Materials and Methods).

[b]Macrophages were obtained from C3H mice injected 15 days before harvest with PBS (mock-immunized) or 500 viable Chlamydia elementary bodies. Chlamydia-sensitized mice also received an intraperitoneal injection of 10^6 elementary bodies 5 days before isolation of peritoneal cells. PEC were plated at 2 x 10^5 cells per well. Numbers shown represent cmp ^{125}I-protein A bound per 10^5 cells. Results of a single representative experiment are shown.

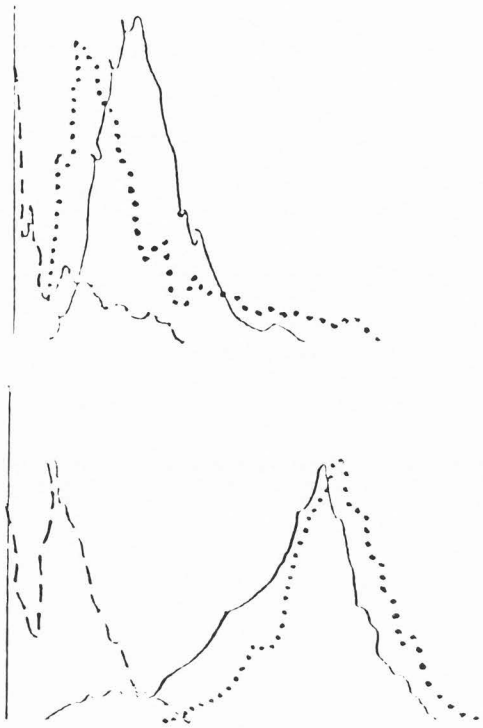

Figure 2. Immunofluorescence staining of control (upper panel) and Chlamydia-elicited peritoneal macrophages (lower panel) as detected by monoclonal anti-I-A^k antibody 10-2.16 (·····) and with monoclonal anti-H-2^k antibody 16-1-2 (——). Staining of the cells with monoclonal anti-I-E^k antibody gave a profile which is superimposable on that obtained using the anti-I-A^k antibody. Also shown in both panels is staining of the peritoneal macrophages with a control monoclonal anti-I-A^d antibody, MK-D6 (-----). Cells were stained directly with fluorescein - conjugated antibodies and were amplified on an Epic-C flow cytometer with a logarithmic amplifier. The fluorescence intensity axis spans 3 decade logs. Since both the I-A and H-2 profiles in the immune cells cover a broad range of fluorescent intensities, these macrophages are heterogeneous with respect to expression of class I and II MHC antigens.

these cell populations; however, with this method, it is not possible to determine the relative contribution of cells within the population which are expressing high and low antigen levels.

Cytofluorometric analysis was used to examine the population in more detail in order to determine the distribution of histocompatibility antigen expression on cells within the populations. As shown in Figure 2, the profiles obtained by analysis of cells labeled with antibody and FITC-conjugated protein A indicate a broad range of expression of Ia (·····) and H-2 antigens (——) within both cell populations, indicated by profile distribution extending over the three-decade log scale of fluorescence intensity. In addition, a comparison of the profiles of macrophages from mock-immunized controls (Fig. 2, upper panel A) and those from immunized animals (Fig.2, lower panel B) reveals that those from immunized animals express increased levels of H-2 and Ia antigens, indicated by a shift to the right on the fluorescence intensity axis. Staining of the cells with monoclonal anti-I-E antibody gives a profile which is superimposable on that obtained using the anti-I-A^k

antibody (data not shown).

As with analysis by RIA, flow cytometry provides a rapid method of determining relative levels of surface molecules on a large number of cells; however, with this type of analysis additional information can be obtained. The measurement of the fluorescence of individual cells can be used to generate a histogram which illustrates the relative number of cells in the population (vertical axis) which display a given level of fluorescence (horizontal axis) (Loken and Stall, 1982). In addition, logarithmic presentation of the fluorescence signals, as indicated here, allows clear-cut distinction of cell populations.

In order to explore further the heterogeneity of Ia antigen expression on individual cells, we then turned to SEM analysis. This technique allows the rapid viewing of the surface of a large number of intact cells. The resulting 3-dimensional image is then used to examine the surface expression of histocompatibility antigens on macrophages of a particular morphological type. The colloidal gold markers used to visualize the Ia and H-2 glycoproteins were small enough (24 nm) to provide important information as to the exact localization of these antigens in relation to individual cell surface microprojections.

Among the peritoneal cells from infected mice, only the extremely flat macrophages with few membrane ruffles or microvilli express high levels of H-2K,D and I-A/I-E antigens (Figs. 3-6). Both classes of surface glycoproteins occur in a monodispersion over the entire cell surface. In contrast, the extensively ruffled macrophage subpopulation displays very low levels of I-A/I-E molecules and moderate levels of H-2K,D antigens (Fig. 7). Peritoneal macrophages from uninfected mice also express moderate levels of class I and low amounts of class II MHC antigens (Fig. 8). Thus, the scanning electron micrographs clearly show that Chlamydial infections result in greater numbers of Ia-positive cells in the peritoneum, as well as an increase in class II molecules/cell. Further, SEM reveals that levels of class II , but not of class I, antigens correlate with the morphological subtype of the macrophage.

Discussion

In this study we compare the information which can be obtained from analysis of macrophage histocompatibility antigen expression by the techniques of radioimmunoassay, flow cytometry, and scanning electron microscopy. Each technique provides distinct information concerning the display of these antigens either at a population or a single cell level. For example, analysis of surface histocompatibility antigens with RIA determines the relative number of antigens per given number of cells. Our initial results revealed an increased level of expression, relative to control cells, of both class I and class II antigens. These results, however, give no indication of the heterogeneity of antigen expression within the immune cell population. To identify cell populations expressing varying levels of antigen expression we further analyzed the macrophages by flow cytometry. With this technique, one can examine individual cells and determine the relative level of fluorescein-labeled antigens on the cell surface. Our analysis revealed that there are more Ia-positive macrophages in the peritoneum of Chlamydia infected mice and that there are more class II molecules/cell. This type of analysis thus gives an indication of the degree of heterogeneity within the cell population.

Finally, scanning electron micrographs of cells from infected mice were examined to determine if a correlation between macrophage morphology and histocompatibility antigen levels could be observed. These results demonstrated that a particular macrophage morphology (flat with few surface ruffles or villi) is associated with high levels of both class I and II antigens. Other morphological subtypes present in the peritoneal macrophage population expressed moderate levels of H-2K,D and low levels of Ia antigens.

Increased expression of surface Ia antigens has been observed on macrophages isolated from animals infected with a variety of organisms (Listeria monocytogenes, Trypanosoma cruzi, and Toxoplasma gondii), suggesting that this surface marker correlates with aspects of macrophage activation during microbial infection.

These studies demonstrate that SEM analysis of cell populations can provide unique information concerning the display of cell surface histocompatibility antigens on cells of the mononuclear phagocyte lineage and further suggest that this technique may be applicable to study of the cell surface mosaic of a variety of cell types. With respect to macrophages, this technique may be particularly useful in fine dissection of the process of macrophage activation occurring during development of an immune response. This hypothesis is strengthened by our observation of significant differences in Ia (class II) antigen display between macrophages activated in situ by Chlamydial

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Figure 3. A uniformly spread macrophage from immunized mice characterized by high levels of class II MHC antigens. Cell is labeled with monoclonal anti-I-E^k antibody, 14-4-4, and protein A-Au. Labels are distributed over the entire cell surface. The arrow indicates the location of the enlarged area depicted in the following micrograph. Bar = 10 μm.

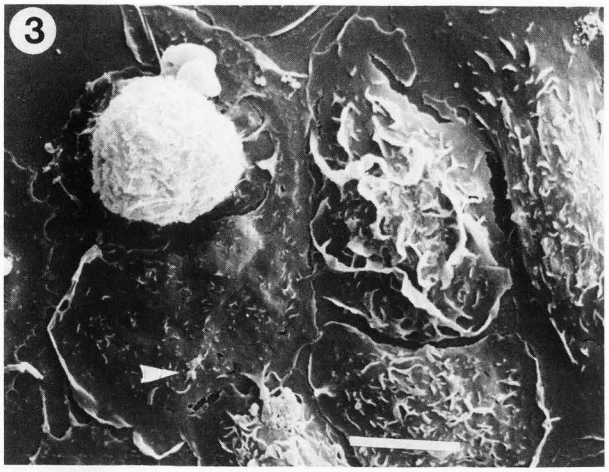
Figure 4. Higher magnification of the cell in Fig. 3 shows the distribution of I-E antigens on the macrophage surface. Bar = 1.0 μm.

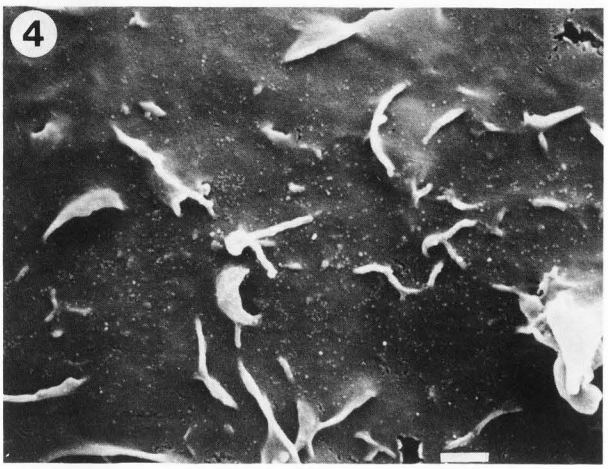
Figure 5. The well-spread, highly Ia-positive macrophages from immune mice also express high levels of H-2 antigens. Arrow indicates the surface area which is enlarged in the following micrograph. Bar = 1.0 μm.

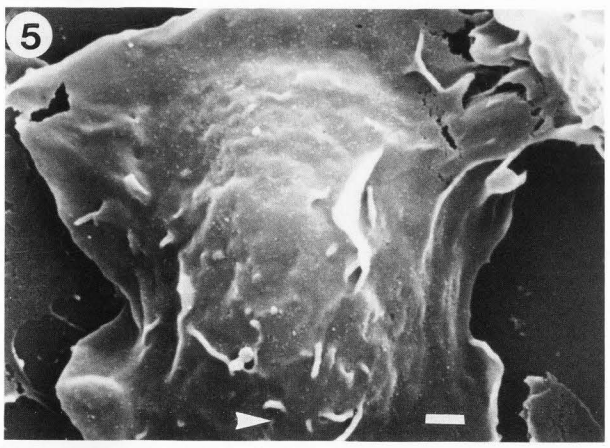
Figure 6. A higher magnification of the macrophage in Fig. 5 shows labeling with the anti-H-2K,D antibody 16-1-2. Bar = 1.0 μm.

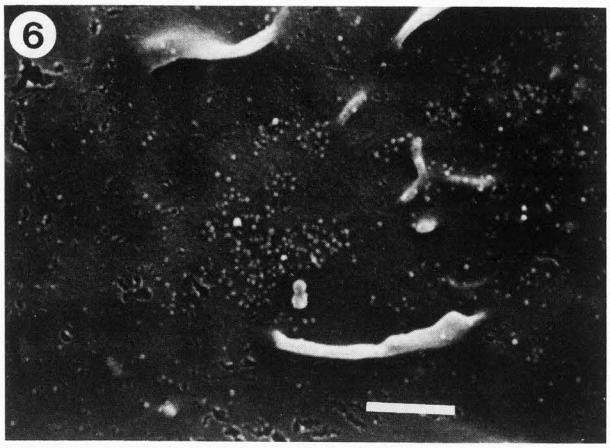
Figure 7. Ruffled peritoneal macrophages from Chlamydia-sensitized mice express low or moderate levels of class II MHC antigens. Bar = 1.0 μm.

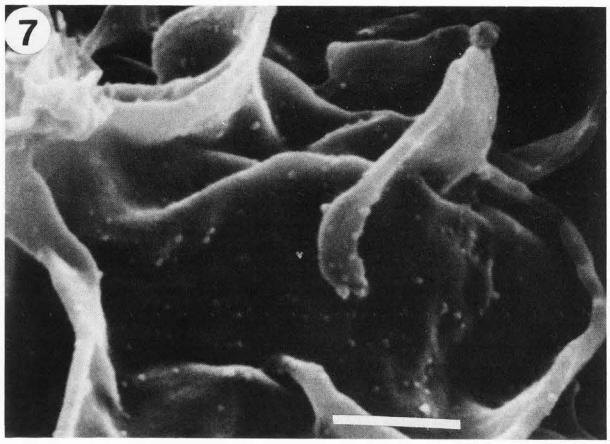
Figure 8. In contrast to the highly Ia-positive spread cells from immunized mice, a similarly spread macrophage from nonsensitized mice shows very few anti-I-E^k labels. Bar = 1.0 μm.

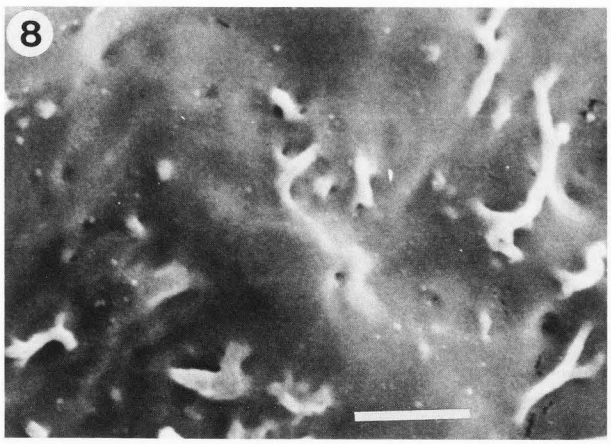












infection and those activated in vitro by immune interferon (Guagliardi, et al., manuscript in preparation).

This study demonstrates an integrated approach to the study of macrophage activation utilizing RIA, flow cytometry, and scanning immunoelectron microscopy. We feel that quantitation of surface antigens can be done rapidly on a large number of cells using flow cytometry and RIA. Scanning electron micrographs, on the other hand, were successfully employed to demonstrate a correlation between macrophage morphology and histocompatibility antigen expression. In addition, we were able to map the location of these antigens on the macrophage surface. One extension of this technique which was not approached in these studies, would be the actual quantitation of gold beads

(and thus antigen quantitation) on a per cell basis. The extremely low nonspecific labeling seen with the antibodies directed against surface antigens of the inappropriate haplotype and the fact that these are all surface antigens, and thus, accessible to antibody, would be positive factors in such quantitative studies. Although many technical aspects must be considered for this type of analysis, including the concentration of gold markers, temperature, medium viscosity, size of gold markers, and staining time, such studies would provide additional information concerning antigen distribution on morphologically distinct cell populations and will be an emphasis of future work from our laboratory. (Park et al., in press)

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Discussion with Reviewers

K. Ozato: Why were the numbers of colloidal gold per cell (or per unit area) not enumerated and the tabulation of this data presented?
D. Handley: Why did you not quantitate the surface density of the gold label?
Authors: We feel that quantitation of surface antigens can be done rapidly on a large number of cells using flow cytometry/RIA. Scanning electron micrographs, on the other hand, were employed in this study to determine if there is a correlation between macrophage morphology and histocompatibility antigens and to discern whether there is an association of these antigens with a particular structure or location on the macrophage surface. The quantitation of gold beads on a per cell basis may be possible; however, this was not attempted in these experiments for this type of analysis.

E. de Harven: Why have the authors chosen the protein A ligand for binding to the Fc fragments of the murine monoclonal antibodies?
Authors: Protein A can bind 1-2 monoclonal antibodies, whereas an antimouse immunoglobulin could bind a maximum of 7-8 such antibodies. The use of protein A, therefore, leads to less "amplification" than a species specific antibody. The use of a directly gold-conjugated primary antibody would be the method of choice for the best spatial resolution of the membrane antigen sites; however, we have found some of the monoclonal antibodies, especially those with high isoelectric points, to have reduced affinity when coupled directly to gold.

H. Gamliel: Given the well-documented effects of glutaraldehyde fixation on surface antigens, did you follow changes in MHC antigen expression resulting from different fixatives or diverse concentrations/duration of glutaraldehyde fixation?
Authors: We have compared the degree of labeling on unfixed and fixed (2% and 0.2% glutaraldehyde) macrophages and found no difference in the quantity or distribution of histocompatibility antigens on the cell surface. Treatment of the cells with 5% glycerol (in hepes buffer) before dehydration was more effective than glutaraldehyde postfixation in preserving the membrane integrity of the extremely flat macrophages; cells of this particular morphology are more sensitive to damage during the dehydration and critical point drying process than are cells of other morphological subtypes.

E. de Harven: How do you know that the small round cells in the micrographs are macrophages?
Authors: The identity of adherent peritoneal cells was confirmed by RIA analysis. Results of experiments in which cell monolayers were labeled with α-Thy 1.2 or α-MAC-1 supernatants and ^{125}I-protein A indicate that the adherent cells possess the MAC-1^{+} Thy 1.2^{-} phenotype characteristic of cells in the mononuclear phagocyte lineage.

E. de Harven: Were similar differences in labeling density confirmed and quantitated by observation of the same cell in backscattered electron imaging mode?
Authors: We were not attempting to compare quantitatively the expression of MHC antigens on activated macrophages. Rather, we are comparing the relative abilities of three techniques to discern heterogeneity in the expression of these cell surface molecules by macrophages. Similar differences in labeling density were observed on the same cells examined in the backscattered imaging mode. This would be the method of choice where quantitation was the principal goal. We have recently found, however, that imaging at 2kV with high resolution low voltage SEM obviates the need for BSE detection of the beads and permits rapid reliable identification by SEM of beads in the 5nm range.

D. Handley: How did you determine the fold-increases in MHC antigens utilizing RIA as stated in the text?
Authors: The "fold increase" in class II antigens described in Table 1 are obtained by the following calculation:

$$\frac{\text{cpm immune-boosted macrophages}}{\text{cpm mock-immunized macrophages.}}$$

D. Handley: In the RIA, how were the relative numbers of antigens per given number of cells determined?
Authors: The numbers shown in Table 1 represent cpm ^{125}I-protein A bound per 10^5 cells. The cell number was determined from an assay which utilizes the binding of A Hoechst dye for quantitation of cellular DNA (Labarca and Paigen, 1980).

D. Handley: What is the morphological significance of the change in macrophages following immune stimulation?
Authors: That is unknown at present.

H. Gamliel: What are the advantages of preparing one's own particles instead of purchasing a commercial preparation?
Authors: Aside from money-saving reasons, we have found that, by preparing our own gold particles, we can obtain higher concentrations of beads for optimal labeling. The beads produced in our laboratory are qualitatively equivalent to commercially available reagents.