Comparison of Polymorphonuclear Cells from Healthy Donors and Differentiated HL-60 Cells as Phagocytes in an Opsonophagocytic Assay Using Antigen-Coated Fluorescent Beads

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Received 3 September 1999/Returned for modification 9 November 1999/Accepted 8 December 1999

Polymorphonuclear cells (PMNs) from healthy donors and differentiated HL-60 cells were compared in an opsonophagocytic assay using fluorescent latex beads coated with Streptococcus pneumoniae polysaccharide conjugates. Serum-specific phagocytosis was efficiently mediated by both sources of cells, as measured by flow cytometry, but the mean number of beads ingested per cell was three- to fivefold higher when PMNs were used than when HL-60 cells were used. Nevertheless, differentiated HL-60 cells could be a convenient and standardized source of cells to evaluate the functionality of specific antibodies to vaccine candidates as a coating on fluorescent beads.

In the search for new vaccines, investigators often have to select a few candidates among a large number of antigens and formulations. A good way to evaluate the potential of such candidates is to measure the specific immune responses against the different antigens in convalescent-phase or resistant hosts after natural infection with the corresponding pathogen. Besides classical enzyme-linked immunosorbent assay techniques, functional assays, such as opsonophagocytic assays, have been demonstrated to be more relevant to analyze the role of antibodies in protection, in particular in the case of Streptococcus pneumoniae (pneumococcal) or Neisseria meningitidis (meningococcal) infections (1–5). An opsonophagocytic assay using fluorescent beads coated with different antigenic structures has been developed by A. Lehmann and coworkers (1), with polymorphonuclear cells (PMNs) from healthy donors as effector cells; opsonophagocytic activity is measured by flow cytometric analysis, the end points being the percentage of fluorescent cells, the mean number of beads per each phagocyte (designated by M in this study), and the product of these two values, the phagocytosis product (PP) (2, 3). In parallel, the group of G. M. Carlone has developed opsonophagocytic assays using differentiated HL-60 cells. In this assay, live bacteria and, more recently, fluorescently labeled and fixed bacteria were used (4, 5). Opsonophagocytic activity is measured in the former case by viable count, while in the latter case, flow cytometry is used.

In the present study, we evaluated the combined use of antigen-coated fluorescent beads as targets and HL-60 cells as phagocytes compared to PMNs from healthy donors. Streptococcus pneumoniae polysaccharides from serotypes 4 and 14 conjugated to tetanus toxoid (TT [Pn4-4T and Pn14-4T, respectively]) were used as antigens. The assay was set up with rabbit positive sera (demonstrated in the department to be opsonic in human cells in a viable opsonophagocytic assay) and negative sera (directed against an irrelevant conjugate) in order to have well-identified negative controls, because it was difficult to obtain and select human sera without specific anti-pneumococcal antibodies. Nevertheless, several human sera, including a reference serum from Sandoz (Sandoglobuline; Sandoz, Rueil Malmaison, France), were tested in a second step. Rabbit sera heated at 56°C were obtained from animals hyperimmunized with Pn4 polysaccharide coupled to diphteria toxoid (DT) instead of TT to avoid unwanted reactions against the carrier protein (anti-Pn4-DT [positive serum]), or hyperimmunized with an irrelevant Haemophilus influenzae type b (HIB) polysaccharide conjugate (anti-HIB [negative serum]). Antigens and sera were prepared in the Research and Development facilities of Aventis Pasteur in Marcy l’Etoile, France. S. pneumoniae serotype 4 bacteria were heat inactivated (1 h at 60°C) and labeled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, Mo.) to be used as a positive antigen control in the assay. Conjugates were adsorbed to fluorescent beads (Fluoresbrite Plain Microspheres; Polysciences, War- rington, Pa.) with diameters of 1, 3, and 6 μm, as described in reference 1, and the coating efficiency was estimated by protein dosage in the supernatant (bicinchoninic acid protein assay) to be about 20%; despite the evaluation of several different conditions, it has not been possible to reach higher coating values. PMNs were obtained from healthy donors by using Polymorphrep (Nycomed, Oslo, Norway), while HL-60 cells were obtained from the American Type Culture Collection and differentiated as described by Romero-Steiner et al. (5). The general conditions of the assay were based on those described in references 1 to 3 when fluorescent beads and PMNs were used combined with those described in references 4 and 5 when HL-60 cells were used. However, some modifications were introduced, and the influence of different parameters was evaluated, including time of opsonization (from 5 to 45 min), time of phagocytosis (from 15 to 45 min), amount of exogenous human complement serum (Sigma; reference no. S1764 [not reactive against pneumococcal antigens in our assays]), and amount of specific antisera. In addition, labeling of PMNs with anti-CD13-phycocerythrin (PE) (labeling nonlymphocytes, granulocytes, and monocytes) and of HL-60 cells with anti-CD32-PE (labeling differentiated HL-60 cells) fluorescent antibodies (Immunotech, Marseille, France) allowed a more accurate determination of the gates in the flow cytometric analysis. This was performed on a FACScan fluorescence-ac-
FIG. 1. Flow cytometric analysis of opsonophagocytic activity mediated by PMNs (A) or HL-60 cells (B) in the presence of Pn4 bacteria (Bact.), Pn4-TT-coated beads (B+), anti-Pn4-DT serum (S+), or anti-Hib.

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TABLE 1. Percentages of fluorescent cells, M, and PP determined after flow cytometric analysis as presented in part in Fig. 1

| Expt | 1-μm-diam beads | 3-μm-diam beads | FITC-labeled Pn4 bacteria | PMNs | HL-60 cells |
|------|-----------------|-----------------|--------------------------|------|-------------|
|      | S+ | S- | B+ | B- | B+ | B- |                  |                  |
| a    | +  | +  | +  | +  | -  | -  | +                 | 94 (89 ± 7.3)    |
|      | -  | +  | -  | -  | -  | -  | +                 | 7 (11.8 ± 4.6)   |
|      | +  | -  | +  | +  | -  | -  | +                 | 14.5 (16.3 ± 3)  |
|      | -  | +  | -  | +  | -  | -  | +                 | 10 (24 ± 8)      |
| b    | +  | +  | +  | +  | -  | -  | +                 | 62 (57.6 ± 4.5)  |
|      | -  | -  | -  | -  | -  | -  | +                 | 2 (2.6 ± 1.3)    |
|      | +  | -  | +  | +  | -  | -  | +                 | 4 (5.4 ± 2)      |
|      | -  | -  | +  | -  | +  | +  | +                 | 9 (7.9 ± 3.6)    |
| c    | +  | +  | +  | +  | -  | -  | +                 | 32               |
|      | -  | -  | -  | -  | +  | +  | +                 | 2.5              |
|      | +  | -  | -  | -  | +  | +  | +                 | 6.5              |
|      | -  | -  | +  | -  | -  | +  | +                 | 10               |
| d    | +  | +  | +  | +  | -  | -  | +                 | 29               |
|      | -  | -  | -  | -  | +  | +  | +                 | 11               |
|      | +  | -  | -  | -  | +  | +  | +                 | 7                |
|      | -  | -  | +  | -  | +  | -  | +                 | 11.5             |
| e    | +  | -  | +  | -  | +  | -  | +                 | 98 (96 ± 2.5)    |
|      | -  | -  | -  | -  | +  | +  | +                 | 12 (6.7 ± 3)     |
|      | +  | -  | -  | -  | +  | -  | +                 | 57 (38.3 ± 18.5) |
| f    | +  | -  | -  | -  | +  | -  | +                 | 3 (3 ± 0)        |

% Fluorescent cells

M

PP

a Experiments were carried out in presence of Pn4 bacteria (Bact+), Pn4-TT-coated beads (B+), Pn14-TT-coated beads (B−), anti-Pn4-DT serum (S+), or anti-HiB conjugate-negative serum (S−). PMNs or HL-60 cells were used with 1-μm beads (a and b, respectively), 3-μm beads (c and d, respectively), or FITC-labeled bacteria (e and f, respectively).

b Values in parentheses are means ± standard deviations.
cells in some experiments (data not shown). Table 1 also shows that 1-μm-diameter beads were more efficiently and more specifically ingested than 3-μm-diameter beads, which gave a high background level in negative controls; unlike 1-μm beads, 3-μm beads, inducing clearly less favorable results, have not been used extensively (only two independent experiments) with HL-60 cells, and standard deviations are thus not presented in the table for these beads. Six-micrometer-diameter beads were not extensively tested due to the very low level of phagocytosis observed in preliminary experiments (data not shown).

Different human sera, as well as a reference serum from Sandoz (Sandoglobuline), were used at least twice (with similar results) in independent experiments with HL-60 cells. Table 2 shows that 41 to 53% fluorescent cells were obtained according to the sera in the presence of Pn14-TT-coated beads, and phagocytosis products were in the same range of magnitude (mean of about 50%). The reactivity of these sera was less important in the presence of Pn4-TT-coated beads (from 5 to 21% fluorescent cells), as with the corresponding FITC-labeled Pn4 bacteria (from 11 to 16% fluorescent cells). Unfortunately, we did not have access to pre- and postexposure or pre- and postvaccination human sera, and no real negative serum could be used in these experiments, as in the previous ones with rabbit sera.

In conclusion, our results indicate that HL-60 cells can ingest fluorescent beads coated with antigenic preparations in the presence of specific antisera. Although the number of beads ingested was higher when PMNs from different donors were used, HL-60 cells presented less background activity. All in all, the good specificity of the assay should allow the use of this more convenient and standardized HL-60 cell line to screen a potentially large number of antigens and corresponding antisera in a functional assay, in particular if a higher coating efficiency can be reached with these antigens. This would be of great value in vaccine research.

We acknowledge Marie-José Quentin Millet for constant support and Jean-Michel Chapsal for providing the pneumococcal conjugates.

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**TABLE 2. Percentages of fluorescent cells, M, and PP determined after flow cytometric analysis as defined in Table 1**

| Serum | Fluorescence for: | % Fluorescent cells | M | PP |
|-------|-------------------|---------------------|---|----|
|       | Pn4 | Pn14 | FITC-labeled | Pn4 bacteria |
| 1     | +   | -    | -             | 8 | 0.4 | 3.2 |
|       | -   | +    | -             | 45 | 1.12 | 50.4 |
|       | -   | -    | +             | 11 | 0.76 | 8.4 |
| 2     | +   | -    | -             | 8.5 | 0.3 | 2.5 |
|       | -   | +    | -             | 37 | 0.8 | 29.5 |
|       | -   | -    | +             | 13 | 0.6 | 8 |
| 3     | +   | -    | -             | 6 | 0.2 | 1.2 |
|       | -   | +    | -             | 43 | 0.8 | 34.4 |
|       | -   | -    | +             | 14 | 0.6 | 9 |
| Sandoglobuline | + | - | - | 21 | 0.38 | 8 |
|       | -   | +    | -             | 50 | 1 | 50 |
|       | -   | -    | +             | 16 | 0.64 | 10 |

* HL-60 cells were used as phagocytes with four different human sera or a pool of sera to opsonize beads (sera 1, 2, and 3 and Sandoglobuline). Pn4, beads coated with Pn4 conjugate; Pn14, beads coated with Pn14 conjugate.