Specific Cell Binding Using Staphylococcal Protein A Magnetic Microspheres

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Abstract □ Protein A, a protein derived from *Staphylococcus aureus*, was incorporated into the matrix of magnetic albumin microspheres. Because staphylococcal protein A binds most subclasses of immunoglobulin G through their Fc portions, immunoglobulin may be rapidly bound to microspheres without chemical coupling agents or a diaminoheptane spacer group. Microspheres so prepared bind specifically to a given cell type when incubated *in vitro* with a heterogeneous cell population. The use of these microspheres as a drug carrier capable of cellular specificity as well as their ability to isolate homogeneous cell populations rapidly is discussed.

Keyphrases □ Carriers, drug—magnetic microspheres synthesized with immunological specificity, incorporation of staphylococcal protein A into microsphere matrix, cell binding studies *in vitro* □ Staphylococcal protein A—incorporation into microsphere matrix, immunological specificity □ Microencapsulation—symposium, magnetic microspheres synthesized with immunological specificity through incorporation of staphylococcal protein A, immunoglobulin G–blood cell binding studies

The ability to impart *in vivo* specificity to particulate drug carriers is desirable to restrict efficacious, yet toxic, chemotherapeutic agents to desired tissues. Specificity has been achieved either by magnetic restriction of a particulate drug carrier at an *in vivo* target site (1) or by immunological methods (2). Magnetic localization results in specific regional restriction of the carrier, with the subsequent release of entrapped drug into surrounding tissue; immunological methods of targeting drug carriers are capable of theoretically selecting the desired cell type without necessarily being restricted to a specific body area. Combining these two modalities may prove beneficial in the treatment of metastatic and unresectable malignancies.

Conventional means of coupling immunoglobulin to solid carriers involve chemical coupling agents (3–5) and require considerable time for chemical reaction and purification of products. In most cases, it also is necessary to have a diaminoheptane spacer group chemically coupled to the carrier prior to the antibody linkage procedure (3, 4). Subsequent orientation of the covalently bound immunoglobulin is random, resulting in only a percentage of immunoglobulin capable of interacting with the desired antigen.

Staphylococcal protein A was incorporated into the matrix of magnetically responsive albumin microspheres in these laboratories. Because staphylococcal protein A can bind the Fc portion of most subclasses of immunoglobulin G (IgG) (6), specific immunoglobulin directed against a desired cell surface antigen can be coupled to the microspheres by a simple nonchemical incubation. This procedure results in the proper molecular orientation of the IgG molecule so that the bound antibody is capable of antigen interaction.

This paper describes the *in vitro* immunological specificity of these microspheres in the separation of chicken red blood cells from a mixture of chicken and sheep red blood cells and, in a different system, the immunoglobulin-bearing lymphocytes from a spleen cell suspension.

EXPERIMENTAL

Preparation of Microspheres—Microspheres were prepared by a modification of methods described previously (7). Essentially, a phase-separation emulsion–polymerization method was employed. A 0.5-ml aqueous suspension containing 190 mg of dry material was made consisting of 66% human serum albumin[1], 19% ferrosoferric oxide[2], and 15% staphylococcal protein A[3]. Cottonseed oil[4] (60 ml) was added to this suspension, and the emulsion was homogenized by sonication[5] for 1 min at 60 w. The homogenate then was added to 200 ml of constantly stirred cottonseed oil at 120–125° for 10 min. Resultant microspheres were washed free of oil by adding excess anhydrous ether[6] and centrifuging[7] for 15 min at 2000×*g*. After the fourth wash, the microspheres were allowed to air dry and were stored at 4° until they were used.

Coupling of Immunoglobulin to Microspheres—Microspheres (0.5 mg) were suspended in 0.2 ml of 0.154 *N* NaCl with 0.1% polysorbate 80[8] (I). To this suspension was added 0.5 mg of normal rabbit IgG, rabbit anti-chicken red blood cells[9], fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin[10], or rabbit anti-rat immunoglobulin[10], and the mixture was incubated at 37° for 40 min. Excess immunoglobulin was removed by centrifugation with excess I at 10,000×*g* for 5 min.

Labeling of Chicken and Sheep Red Blood Cells—Aliquots of chicken or sheep red blood cells were labeled with chromium 51 to assess the extent of the microsphere immunological specificity as well as cell integrity. Labeling was performed by incubating 1×10^8 chicken red blood cells suspended in 0.2 ml of Hanks balanced salt solution containing 2.5% heat-inactivated fetal calf serum with 100 μCi of sodium [^{51}Cr]-chromate[11] for 90 min at 37°. Sheep red blood cells were treated similarly except that they were incubated overnight at 37°.

Separation of Cells—Microspheres bearing immunoglobulin directed against specific cellular antigens were used to isolate cells exhibiting these antigens from heterogeneous cell populations utilizing the magnetic property of the microspheres (8).

Red Cell Separation—Microspheres (0.5 mg) bearing either rabbit anti-chicken red blood cells or normal rabbit IgG were suspended in 0.2 ml of I. To this suspension was added a mixture of 1×10^6 chicken red blood cells and 1×10^6 sheep red blood cells in 0.2 ml of Hanks balanced salt solution. Depending on the experimental group, either the chicken or sheep red blood cells were labeled with chromium 51. After incubation with the microspheres for 30 min at 37°, the cells were exposed to a 4000-gauss (gradient = 1500 gauss/cm) magnetic field applied to the side of the reaction vessel. Both the supernatant and pellet fractions were counted for chromium 51 γ-emission[12]. Control labeled cells incubated in I were counted for chromium 51 to assess spontaneous release.

Lymphocyte Separation—A suspension of spleen cells was obtained

[1] Sigma Chemical Co.
[2] Magnetite (Fe_3O_4) in aqueous suspension, Ferrofluidics Corp.
[3] Sigma Chemical Co.
[4] Sargent-Welch.
[5] Branson sonifier model 185.
[6] Mallinckrodt Chemicals.
[7] Sorvall model RC-5.
[8] Tween 80, Sigma Chemical Co.
[9] Cappel Laboratories.
[10] The IgG fraction was predominantly anti-IgG as determined by immunoelectrophoresis, Miles Laboratories.
[11] $Na_2{}^{51}CrO_4$, 1 mCi/ml, Amersham.
[12] Beckman model 8000 γ-counter.

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Table I—Separation of Chicken Red Blood Cells (CRBC) and Sheep Red Blood Cells (SRBC) Using Rabbit Anti-Chicken Red Blood Cells Coupled to Staphylococcal Protein A Magnetic Microspheres

Antibody Coupled to Microspheres	Type of Cell Suspension	Percent of ^{51}Cr-Labeled Red Blood Cells Bound to Microsphere	Percent of CRBC in Supernate
Rabbit anti-CRBC (IgG fraction)	^{51}Cr-labeled CRBC (1×10^6) and SRBC (1×10^6)	97.8	0.26
Rabbit anti-CRBC (IgG fraction)	^{51}Cr-labeled SRBC (1×10^6) and CRBC (1×10^6)	9.5	0.33
Normal rabbit IgG	^{51}Cr-labeled CRBC (3×10^6) and SRBC (1×10^6)	10.0	90

by teasing rat spleen (Lewis rats) in Hanks balanced salt solution with 10% heat-inactivated fetal calf serum. After washing three times with Hanks balanced salt solution, the cells were overlaid on Ficoll–Hypaque (specific gravity of 1.072) and centrifuged at 1200×*g* for 25 min at 25° to eliminate the red blood cells and nonviable monocytes. The resultant interface band was removed and assessed for viability by trypan blue dye exclusion.

Rabbit anti-rat immunoglobulin, normal rabbit IgG, and rabbit anti-chicken red blood cells were coupled to 0.5 mg of staphylococcal protein A microspheres (0.5 mg of IgG/0.5 mg of microspheres) as described previously. Splenocytes (2×10^6) suspended in Hanks balanced salt solution with 2.5% heat-inactivated fetal calf serum were added to the IgG-bearing microspheres. Mixtures were incubated for 2.5 hr at 4°. Cells with adherent microspheres were separated magnetically, and resultant cells in the supernates were analyzed for viability. The number of residual immunoglobulin-bearing cells was determined by incubating cells in the supernate for 20 min at 37° with fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin.

Immunological Sensitivity—The sensitivity of the microspheres for cellular interaction and subsequent magnetic removal from suspension was tested in two systems. Serial dilutions of microspheres bearing rabbit anti-chicken red blood cells were made, and 1×10^6 chicken red blood cells labeled with chromium 51 were added to each dilution. Incubation of the microspheres and cells was carried out for 30 min at 37°. Cells with adherent microspheres were removed magnetically, and both pellet and supernatant fractions were assessed for chromium 51 activity.

In addition, serial dilutions of the microspheres coupled with rabbit anti-human IgG[13] were made, and 5×10^6 human peripheral lymphocytes were added to each dilution[14]. The mixture was incubated for 1 hr at 4°, after which cells with adherent microspheres were removed magnetically. Supernatant cells were assessed for the presence of surface immunoglobulin by incubation with fluorescein isothiocyanate-conjugated rabbit anti-human IgG[13] for 20 min at 37° and analyzed by fluorescence microscopy.

RESULTS AND DISCUSSION

A qualitative assessment of microsphere capacity for IgG binding was determined by incubating staphylococcal protein A microspheres with fluorescein isothiocyanate-conjugated rabbit and anti-rat immunoglobulin. The apparent diffuse surface fluorescence of the microspheres suggested that staphylococcal protein A was available on the microsphere surface for IgG binding. Moreover, despite the fact that staphylococcal protein A was an integral part of the microsphere matrix, it was oriented on the microsphere surface in such a manner as to retain its ability to bind IgG, as determined by the presence of fluorescence.

Results of the red cell separation are shown in Table I. Using ^{51}Cr-labeled chicken red blood cells, when a mixture of 1×10^6 chicken red blood cells and 1×10^6 sheep red blood cells was incubated with 0.5 mg of staphylococcal protein A microspheres bearing rabbit anti-chicken red blood cells, 97.8% of the chromium 51 label was associated with the cells removed magnetically from suspension. The percentage of residual chicken red blood cells remaining in the supernate after magnetic isolation of cells was 0.26%, as judged by light microscopy. Nonspecific adherence of sheep red blood cells was determined by labeling them with chromium 51. Based on the chromium 51 counts, 9.5% of the label was associated with the microsphere fraction after magnetic separation of the mixture.

In addition, when normal rabbit IgG was coupled to the microspheres, an immunoglobulin unrelated to either chicken or sheep red blood cells (again 10% nonspecific adherence) was present. This minimal nonspecific adherence may be due to bound cross-reactive rabbit IgG based on shared antigens between microbes and mammalian cells (9). Because of the selective interaction of the anti-chicken red blood cell-bearing microspheres for chicken red blood cells, a highly purified and enriched sheep red blood cell population was generated.

Similar good specificity and separation were obtained when microspheres were used to separate immunoglobulin-bearing lymphocytes from a suspension of spleen cells. Between 43 and 51% of the starting splenocytes were immunoglobulin bearing, as determined by fluorescein isothiocyanate-conjugated anti-rat immunoglobulin binding to cells. When rabbit anti-rat immunoglobulin was coupled to staphylococcal protein A microspheres and subsequently incubated with 2×10^6 splenocytes, only 0.5% of immunoglobulin-bearing splenocytes remained in the supernate after magnetic removal of cells with adherent microspheres (Fig. 1). Moreover, when these microspheres were incubated with 2×10^6 thymocytes (normally containing 4–6% immunoglobulin-bearing lymphocytes), no immunoglobulin-bearing cells could be detected in the supernate representing 100% separation of immunoglobulin-bearing cells from nonimmunoglobulin-bearing cells. No loss of cellular viability was noted in any experimental group. To control for nonspecific adherence, normal rabbit IgG and rabbit anti-chicken red blood cells were coupled to the microspheres and incubated with the splenocytes. After magnetic separation of cells, 47 and 44% of the splenocytes in the supernate were

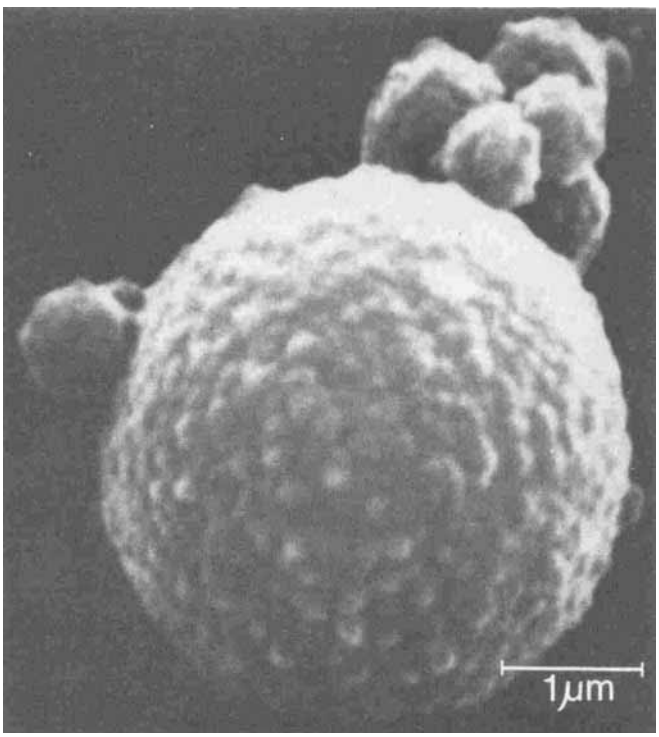

Figure 1—*Scanning electron micrograph of a rat lymphocyte with adherent microspheres (1 μm) on its surface. The micrograph is representative of cells obtained after coincubation of staphylococcal protein A microspheres bearing rabbit anti-rat immunoglobulin and splenocytes and subsequent magnetic isolation of microsphere–cell complexes.*

[13] Antiheavy and light chains, Cappel Laboratories.

[14] Lymphocytes were prepared similarly to the rat splenocytes; 15–30% of circulating peripheral lymphocytes are immunoglobulin bearing.

immunoglobulin bearing, respectively. These figures correlate well with the known number of immunoglobulin-bearing splenocytes (44–51%) and demonstrate minimal nonspecific adherence.

To determine the immunological sensitivity of the microspheres, two different systems were tested. It was found that 100% of the chicken red blood cells was magnetically removed from suspension when ≥ 104 μg of microspheres was incubated with 1×10^6 chicken red blood cells. One milligram of staphylococcal protein A microspheres coupled with rabbit anti-human IgG effectively depleted all immunoglobulin-bearing human peripheral lymphocytes from a suspension containing $\leq 80 \times 10^6$ cells. These values demonstrate the capacity of staphylococcal protein A microspheres to separate a large number of cells rapidly and effectively *via* immunological means.

The ability of staphylococcal protein A microspheres to bind several different antiserums, which can confer a high degree of immunological specificity *in vitro*, was presented in this paper. Because magnetic microspheres can be used as drug carriers (1), it is proposed that with the addition of staphylococcal protein A to the microsphere matrix, tumor-specific antibody could be bound rapidly, resulting in a carrier capable of area-specific drug delivery and tumor-cell specificity. Thus, drug action could be limited solely to the desired cell population.

Because antibody coupling is rapid and easily performed, it is ideally suited for cell separation. No spacer group has to be coupled to the microspheres prior to antibody coupling. Moreover, no chemical coupling agents are used, which allows the reutilization of valuable antiserums. Since most IgG subclasses can bind staphylococcal protein A microspheres, this system may be valuable in rapid protein and enzyme purification. This system also may be useful as a mechanism for automated radioimmunoassays.

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In Vivo Kinetics of Magnetically Targeted Low-Dose Doxorubicin

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Abstract □ The *in vivo* kinetics of low-dose doxorubicin (0.05 mg/kg), entrapped in a carrier and magnetically targeted, were characterized in a rat tail model. Tissue concentrations of doxorubicin at a preselected target site and in various organs were followed over time. As late as 60 min postinjection, 3.7 μg/g of drug was found at the target site with no detectable drug levels found in any organ. In comparison, a 100-fold higher dose (5.0 mg/kg iv) of free doxorubicin yielded drug concentrations of 1.8 μg/g at the target site and 15.0 μg/kg in the pooled organs. Therefore, 1% of the free intravenous dose targeted magnetically yielded approximately twice the local doxorubicin concentration at a preselected target site with no detectable systemic distribution. Magnetic targeting of particulate drug carriers to localized disease sites is suggested as an efficient method of obtaining high local drug concentrations and may reduce many unwanted side effects from unrestricted systemic circulation.

Keyphrases □ Microspheres, magnetic—as drug carriers, doxorubicin, *in vivo* □ Carriers, drug—doxorubicin-bearing magnetic microspheres, *in vivo* □ Doxorubicin—*in vivo* fate of localized low dose, kinetics □ Delivery, drug—magnetic microspheres as drug carriers, doxorubicin, *in vivo* □ Microencapsulation—symposium, doxorubicin-bearing magnetic microspheres, *in vivo* fate of localized low dose

Current approaches to enhancing the tumoricidal activity of antineoplastic agents involve combining them with various carriers in the hope of favorably altering their systemic distribution. Early work by Chang (1), who encapsulated proteins and enzymes to develop artificial organs, suggested that drugs also may be encapsulated in carriers. The current targeting of drug carriers such as liposomes (2) or natural cells (3) depends on a moderate to marked enhancement of the endocytic activity (4) exhibited by some solid tumors. Other modalities for enhancing drug concentration at known tumor sites include regional perfusion, hyperthermia, and local installation and implantation of drugs. However, most of these modalities have met with limited clinical success (5).

A biophysical approach to targeting chemotherapeutic agents to known tumor sites was described recently (6). The system utilizes magnetically responsive albumin microspheres as the carrier for entrapped doxorubicin. The application of an extracorporeal magnetic field over a selected body area results in accumulation of the carrier containing the entrapped drug at the target site. The synthesis (7), magnetic responsiveness (8), and *in vivo* distribution (6) of the carrier at one time period postinjection were described previously. However, the correlation of carrier distribution with doxorubicin distribution over time was not examined. In this report, the levels of doxorubicin obtained at different time intervals at a preselected target site are characterized. In addition, a low dose (0.05

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