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Monoclonal antibody-based targeting of methotrexate-loaded microspheres

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Summary

Monoclonal antibodies against common acute lymphoblastic leukemia antigen were produced by mouse tumor induction and their methotrexate-loaded immunomicrospheres were prepared by the glutaraldehyde activation method. The immunomicrospheres showed selective affinity for the antigen-positive human leukemia cell line Daudi and non-specific binding of the cells was less than 3%. The methotrexate-loaded immunomicrospheres curtailed the growth of Daudi cells selectively but exerted only a slight effect on the antigen-negative cell line K562 in vitro. Neither immunomicrospheres lacking methotrexate nor non-immunomicrospheres containing methotrexate were able to suppress to a significant extent the growth of both Daudi and K562 cells in vitro.

Introduction

With interest increasing in the application of monoclonal antibodies to cancer therapy in both native and drug-conjugated forms (Houghton and Scheinberg, 1986; Brodsky, 1988), a great deal of effort has been directed towards the use of monoclonal antibodies to target potent cytotoxic agents. This has occurred because of the increase in their therapeutic index, either by improving the localization of agents within tumors or via the minimization of toxic responses in normal tissues, this being a major limitation in conventional cancer chemotherapy which constitutes a major therapeutic approach to the treatment of cancer (Arnon et al., 1985).

Several experimental procedures have been examined for the monoclonal antibody-based targeting of cytotoxic drugs including drug immunoconjugates (Uadia et al., 1984) and drug-loaded immunoliposomes (Singh et al., 1988).

In this study, we have examined the applicability of cytotoxic drug-loaded immunomicrospheres for use in a new site-specific drug delivery system for cancer therapy. Polymeric immunomicrospheres have been extensively studied for purposes of (1) cell labeling and separation (Rembaum and Margel, 1978), (2) diagnosis and therapy (Suzuta, 1983), and (3) cancer therapy by bone marrow transplantation (Treleaven et al., 1984). The possibility of using immunomicrospheres for drug targeting has been proposed (Illum et al., 1983;

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Rolland et al., 1987) in previous studies, however, no discussion of the situation with respect to the cytotoxic drug-loaded forms was provided.

For a model, monoclonal antibodies (MoAbs) against common acute lymphoblastic leukemia antigen (CALLA) and the folate antagonist, methotrexate (MTX), were adopted as the antibody and cytotoxic drug, respectively. Both antigen-positive and antigen-negative cell lines were employed for in vitro evaluation of MTX-loaded immunomicrospheres.

Materials and Methods

Production and characterization of monoclonal antibodies

A hybridoma, designated CALL269-65, which secretes MoAbs against CALLA, was established from hybrids of Balb/c mouse splenocytes immunized with the human acute lymphoblastic leukemia cell line NALM-6 and the mouse myeloma cell line P3X63-Ag8.V653, according to the methods described previously (Köhler and Milstein, 1975; LeBien and Kersey, 1985). The MoAbs were produced by mouse tumor induction and the IgG fraction of the ascitic fluid of hybridoma-bearing mice was purified by ammonium sulphate precipitation and affinity chromatography on protein-A Sepharose CL-4B (Pharmacia, Sweden).

Antibody specificity for a number of human leukemia cells was determined via binding assays with cultured human leukemia cell lines and by an enzyme-linked immunoabsorbant assay (ELISA) using anti-mouse IgG-HRPO (Bio-Yeda, Israel). Isotypes of MoAbs were determined by Ouchterlony double-diffusion assays.

Preparation of microspheres

MTX-loaded microspheres (MTX-MS) and their magnetic microspheres (MTX-MMS) were prepared according to a modification of a previously reported method (Lee et al., 1988). Briefly, 1 ml of an aqueous mixture of 300 mg bovine serum albumin and 50 mg MTX · HCl (both from Sigma, U.S.A.) with or without 0.2 ml magnetic fluids (W-40, Taiho, Japan) was sonicated in 30 ml isooctane containing 0.1 ml sorbitan monooleate (Sigma) on an ice bath. The resultant emulsion was treated with glutaraldehyde (Sigma) then washed with acetone and vacuum dried. Microspheres (MS) without MTX were also prepared by using the same procedure with aqueous bovine serum albumin solution but without MTX.

Preparation of immunomicrospheres

To prepare MTX-loaded immunomicrospheres (MTX-IMS), 50 mg of MTX-MS were incubated with 10 mg of MoAbs dissolved in 10 ml of Hank's balanced buffer solution (HBSS) at 4 °C for 30 min, washed with cold HBSS and freeze-dried. The same procedure was employed for preparation of MTX-loaded magnetic immunomicrospheres (MTX-MIMS) from MTX-MMS and also immunomicrospheres (IMS) from MS.

Measurement of physicochemical properties of microspheres

The size of microspheres and their distribution were determined by using a scanning electron microscopic technique (Lee et al., 1988). To measure the MTX content in microspheres, 10 mg of microspheres were digested overnight in 1 ml of 0.4% trypsin (type II, Sigma) at 37 °C and centrifuged at 10000 rpm for 10 min, followed by the MTX content in the supernatant being assayed by HPLC (McElnay et al., 1988). The magnetite content of microspheres was determined by atomic absorption spectroscopy (Lee, 1988).

Immunoreactivity of immunomicrospheres

To determine the immunoreactivity of MTX-IMS with cells, the following experiments were performed: (a) difference interference contrast microscopic and scanning electron microscopic observation (Molday, 1983), following MTX-IMS incubation of the cells in HBSS at room temperature for 5 min; and (b) magnetic cell separation as described below followed by incubation of MTX-MIMS with the cell lines in RPMI 1640 (Sigma) medium at room temperature for 10 min.

Cell separation

Following incubation of 10^7 cells in 10 ml RPMI 1640 medium with 10 mg MTX-MIMS, the mix-

ture was loaded into a constant flow magnetic separation apparatus (Lee et al., 1988). The applied magnetic field strength and flow rate were maintained at constant values of 10 kG (Cenco, U.S.A.) and 1.5 ml/min (MP-3, Tokyo Rikakikai), respectively. The numbers of cells both bound in the collecting chamber and eluted in the receiver were counted in a hemocytometer.

In vitro cytotoxicity test

Aliquots (2 ml) of each cell type (1×10^5/ml) in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Sigma) were seeded into 6-well microplates (Bellco, U.S.A.), respectively. Subsequently, varying amounts of MTX-IMS, MTX-MS or IMS were added and incubation performed at 37°C for 5 min with gentle agitation, followed by culturing at 37°C under 5% atmospheric CO_2. The number of viable cells remaining in each of the wells after 3 days was determined either with a hemocytometer or by the trypan blue exclusion method.

Results

Monoclonal antibodies

Purified MoAbs from the ascitic fluid of hybridoma-bearing mice belonged to the IgG1 subclass. From the results obtained in ELISA assays of a number of human leukemia-lymphoma cell lines, the MoAbs were shown to undergo binding reactions with NALM-6, Daudi and RPMI 8402 cells, whereas no reactivity was observed with the K562, MOLT 4 and Jukart cell lines. In this study, Daudi, a Burkitt's lymphoma cell line, and K562, a chronic myelogenous leukemia cell line, were employed as antigen-positive and antigen-negative cells, respectively.

TABLE 1

Physicochemical properties of methotrexate-loaded immunomicrospheres

MTX-IMS, -MS, -MIMS, -MMS: methotrexate-loaded immunomicrospheres, microspheres, magnetic immunomicrospheres and magnetic microspheres, respectively.

Microspheres	Mean diameter (μm)	MTX (μg/mg)	Magnetite (%)
IMS	0.92 ± 0.77	–	–
MTX-IMS	1.08 ± 0.75	22.6	–
MTX-MS	1.15 ± 0.81	21.0	–
MTX-MIMS	1.68 ± 1.03	N.A. *	16.8
MTX-MMS	1.57 ± 0.96	N.A.	15.7

* Not assayed.

Physicochemical properties of microspheres

The particle size, and the drug and magnetite content of MTX-loaded microspheres were evaluated, and the results as listed in Table 1 express the data as means of 5 replicate trials, with the particle sizes being given as means ± S.D. to show their distribution. To conform with the data on MTX-IMS and MTX-MIMS, those for MTX-MS and MTX-MMS were recorded not for the freshly prepared state but for the fully hydrated and freeze-dried forms after treatment similar to that employed in the preparation of immunomicrospheres without antibody.

TABLE 2

Magnetic separation of antigen-positive cells with magnetic immunomicrospheres

Test no.	Initial cell no. ($\times 10^5$/ml)		Microspheres (mg)		Passed cell no. ($\times 10^5$/ml)	% bound cells in magnetic field
	Daudi (antigen-positive)	K562 (antigen-negative)	MTX-MIMS	MTX-MMS		
1	10	–	1.0	–	N.D. *	99 <
2	10	–	–	1.0	9.7	3
3	–	10	1.0	–	9.8	2
4	5	5	1.0	–	4.8	52

* Not detectable.

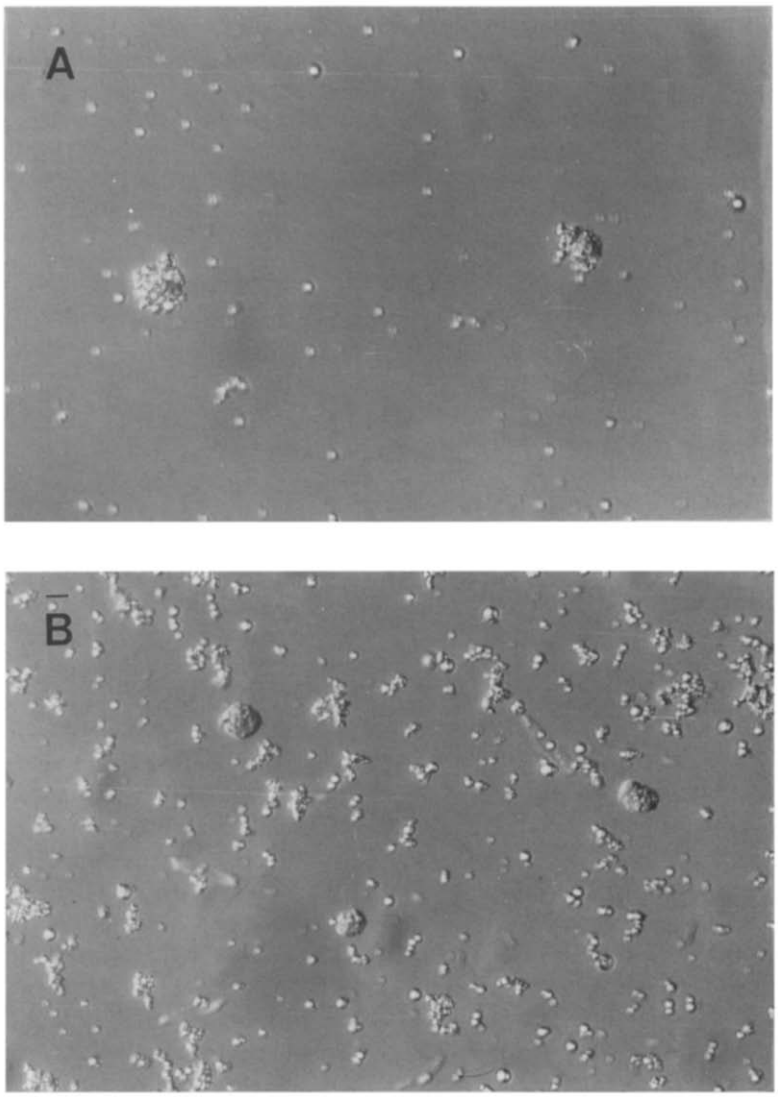

Fig. 1. Differential interference contrast photomicrographs of incubated target cells with methotrexate-loaded immunomicrospheres. (A) Antigen-positive Daudi cells, (B) antigen-negative K562 cells (magnification × 200).

Immunological properties of MTX-loaded immunomicrospheres

MTX-IMS were mixed with Daudi and K562 cells, respectively, and incubated for 5 min. As shown in Fig. 1, MTX-IMS bound selectively at the surface of Daudi cells and adhesion of MTX-IMS to the surface of K562 cells was not observed. Fig. 2 also shows an example of the bound state of MTX-IMS on a Daudi cell in greater detail. Using the same procedure, MTX-MS expressed immunoreactivity with neither Daudi nor K562 cells, while MTX-MIMS and MTX-MMS demonstrated immunoreactivity corresponding to those of MTX-IMS and MTX-MS, respectively.

To determine the efficiency of the selective immune reaction for immunomicrospheres, cells were incubated with MTX-MIMS and MTX-MMS, respectively, and then examined with the magnetic cell separation apparatus giving the results set out in Table 2 where the data are ex-

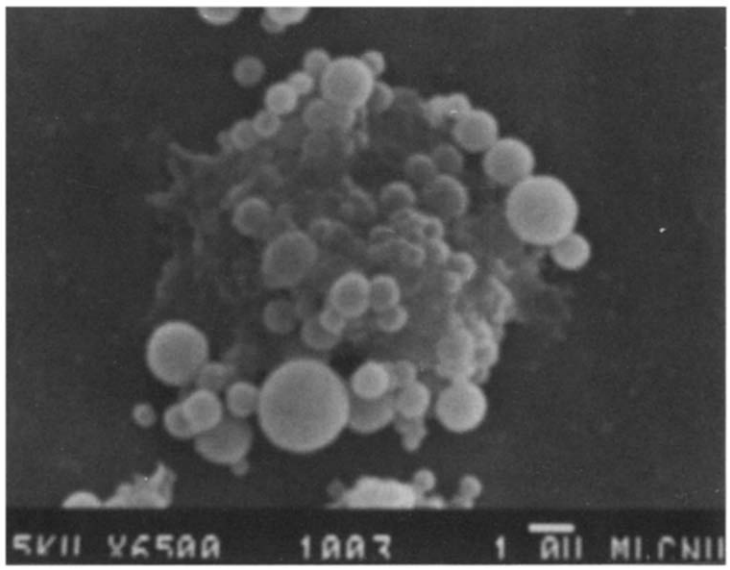

Fig. 2. Scanning electron micrographs of methotrexate-loaded immunomicrospheres attached on the surface of an antigen-positive Daudi cell (Bar: 1 μm).

pressed as the means of 5 duplicate trials. The extent of specific binding of MTX-MIMS on Daudi cells was greater than 99%, whereas the proportion of non-specific binding of MTX-MIMS on K562 cells and of non-specific adherence of MTX-MMS to Daudi cells was no greater than 2 and 3%, respectively. Cell separation could be achieved with an efficiency of 96% in the case of the mixture of Daudi and K562 cells.

TABLE 3

Specific cytotoxicity of methotrexate-loaded immunomicrospheres on human leukemia cells in vitro

Expt. no.	Pre-treatment	Microspheres added (μg/well)	Viable cells/ml ($\times 10^5$) on day 3	
			Daudi (antigen-positive)	K562 (antigen-negative)
1	Control (without medium)	–	4.2	6.1
2	IMS	50	3.6	5.7
		250	3.1	6.0
3	MTX-IMS	50	0.1	4.7
		250	0	3.1
4	MTX-MS	50	3.5	5.3
		250	2.8	4.5

In vitro antitumor activity

As shown in Table 3, in which the data are expressed as means of 5 duplicate cultures, antigen-positive Daudi cells were either killed or their growth arrested by MTX-IMS, the cytotoxicity being dependent on the amount of MTX-IMS added. In contrast, the antigen-negative K562 cells were affected to a lower degree by MTX-IMS as compared with the case for Daudi cells and IMS which contain no MTX but possess equivalent immunoreactivity to MTX-IMS, no significant effect being exerted on the growth of both Daudi and K562 cells. Furthermore, MTX-MS which contains an equivalent amount of MTX but lacks immunoreactivity was found to have a negligible effect on the growth of both Daudi and K562 cells.

Discussion

The most important factors to be considered in the conjugation of monoclonal antibodies to cyto-

toxic drugs or the surface of microspheres concern the necessity of retaining both the initial specific immunoreactivity of monoclonal antibodies and the cytotoxic effect of drugs. In particular, since the entire coupling procedure requires chemical manipulation for achieving conjugation, there is no guarantee of the monoclonal antibodies retaining their immunoreactivity. Therefore, each monoclonal antibody must be individually evaluated for any particular type of drug coupling procedure (Rowland, 1987; Laguzza et al., 1989).

Among the methods considered as a possible process for conjugation of monoclonal antibodies on the surface of microspheres (Illum and Jones, 1985), glutaraldehyde stabilization and activation were adopted for this study.

As shown in Figs. 1 and 2, even after conjugation on the surface of MTX-loaded microspheres, the MoAbs retained the properties of antigen recognition and binding on the antigen-positive cell line Daudi. Furthermore, the high efficiency of cell separation and negligible extent of non-specific or Fc fragment-mediated binding of immunomicrospheres on the cells, as demonstrated in Table 2, support the above assumption. The conjugation method was rather simple and might also be applicable in the case of any immunoglobulin as compared to the use of protein A on albumin immunomicrospheres (Widder et al., 1981; Kandzia et al., 1985), since protein A-coated microspheres bind only protein A-reactive immunoglobulin molecules, thus excluding other antibodies, lectins, or desired conjugates.

Another problem that must be overcome in cytotoxic drug immunoconjugate cancer therapy involves the manner of delivery of a large number of drug molecules per molecule of monoclonal antibody to cancer cells in order to kill such cells without significant reduction in the activity of either the monoclonal antibody or cytotoxic drug (Rowland, 1987). This problem could be resolved by using cytotoxic drug-loaded immunomicrospheres, since a high drug payload in immunomicrospheres is possible to achieve, irrespective of the antibody itself. A further advantage of cytotoxic drug-loaded immunomicrospheres over conventional cytotoxic drug immunoconjugates may well be the release of active and unmodified drug, as no chemical modification is performed on the cytotoxic drug.

The present results as listed in Table 3 clearly indicate that MTX was targeted efficiently to antigen-positive cells. Hence, it could be confirmed that the cytotoxic effect of MTX-IMS is a consequence of antigen-mediated drug-loaded immunomicrosphere targeting and does not require a non-specific, protein carrier-mediated drug delivery phenomenon to be involved as explanation. The factors regulating the potency of drug-loaded immunomicrosphere-based cytotoxicity in vitro may be numerous and complex in nature. The processing of the cytotoxic effect of MTX-IMS in this model study probably involves the initial binding of monoclonal antibody end-conjugated on the surface of MTX-loaded microspheres to the cell surface, followed by the release of MTX from microspheres to yield a high concentration of MTX localized around target cells, and finally, interaction of MTX with target sites for cell inactivation.

Monoclonal antibody-based targeting of cytotoxic drugs to tumor cells has become an increasingly popular idea, although there are basic, serious drawbacks, including a lack of monoclonal antibodies truly specific for the particular type of tumor cells and the heterogeneity of tumor cells when expressing antigens. In addition, it has not been possible to identify any leukemia-specific antigen in human leukemias as another tumor antigen in tumor cells, however, the CALLA is known to be the most important and multiple antigenic marker found thus far in the diagnosis of leukemia (Minowada et al., 1987). Therefore, a monoclonal antibody against CALLA was selected as a model monoclonal antibody in this study.

In conclusion, this study demonstrates that MTX-IMS can be applied in using a monoclonal antibody-based targeting delivery system of cytotoxic drug together with cytotoxic drug-loaded immunoliposomes (Singh et al., 1988) and implies drug-loaded immunomicrospheres or their magnetic immunomicrospheres to be appropriate for bone marrow transplantation by elimination or destruction of cancer cells in vitro (Treleaven et al., 1984).

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