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MODIFICATION OF MONOCLONAL ANTIBODIES BY POLYMERS POSSESSING CHELATING PROPERTIES

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The use of monoclonal antibodies in diagnosis has increased steadily in recent years. Their use is based on the production of corresponding antibodies against characteristic components of a diseased organ or tissue, labeling the antibodies thus obtained with a radioactive or other label, which can be visualized by noninvasive methods, intravenous injection of labeled antibodies into the patient, and subsequent recording of accumulation of the label in the target zone. This approach has already been tested in the diagnosis of myocardial infarction [4], when antibodies against cardiac myosin are used to visualize the zone of necrosis, in the diagnosis of thrombosis [2], when antifibrin antibodies are used, and in the diagnosis of certain tumors [10], when antibodies against components of the tumor cells are used as carriers of the label. To label antibodies, γ-radioactive isotopes (^{111}In, ^{99m}Tc, ^{123}I, ^{131}I) or metals with a strong NMR signal (Mn, Cd) may be used. In the first case, to obtain an image of the affected zone, a Gamma-camera is used; in the second case an NMR tomograph is used. Several methods of binding the label to antibodies have now been developed, the most effective being preliminary modification of the antibodies by groups chelating heavy metals, such as ethylenediaminetetraacetic acid (EDTA) or diethylenetriaminepentaacetic acid (DTPA) [7].

Unfortunately, all existing labeling methods have obvious disadvantages. When the radioactive marker and radioisotope diagnosis with an antibody is used, in principle a sufficient amount of radioactivity can be bound; but to obtain a good picture a long exposure is needed, in order to remove unbound label from the bloodstream, since it is impossible to distinguish the activity specifically bound with the affected zone against such a background. In the case of NMR diagnosis, to obtain a clear picture a high local concentration of the metallic label must be obtained because of the relatively low sensitivity of the method. Since in general it is impossible to bind more than three or four atoms of a metal with one molecule of an antibody (because of loss of activity and specificity by the antibody as a result of "remodification"), to accumulate a sufficient quantity of marker in the required site a large quantity of antibodies has to be injected, and this is highly undesirable. The ideal marker of a pathological zone would be antibodies capable of carrying a large number of atoms of a marker metal per molecule, without losing their activity and specificity in so doing, and one which

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binds rapidly in the zone of the lesion, with simultaneous rapid removal of the unbound fraction from the bloodstream, preferably through the kidneys (with the urine).

This paper describes a basically new approach to obtaining diagnostic antibodies, consisting of a "one-point" modification of the antibody, without loss of its activity, by a high-molecular-weight synthetic polymer with the ability of effectively chelating ions of heavy metals. As a result, preparations of active antibodies containing some tens of atoms of the metal per protein molecule can be obtained.

Monoclonal antibodies R11D10 to human cardiac myosin, also cross-reacting with canine cardiac myosin, were obtained by the method described previously [6] with immunization of BALB/c mice with human cardiac myosin, prepared by the method in [3], and subsequent fusion of spleen cells of the immunized mice with plasmacytoma cells of the SP2/0-Ag14 line [11]. Antibodies of the IgG class were isolated from ascites fluid of BALB/c mice, into which 10^6 hybrid cells were injected, by affinity chromatography on protein-A-Sepharose [1]. Fab-fragments of monoclonal antibodies were obtained by the method in [5]. Activity and specificity of the antibodies and their fragments were tested by solid-phase radioimmunoassay – by direct and indirect methods [5].

Chelating polymers of two fundamental types were used to modify the antibodies and their fragments: polyethyleneimine with molecular weight of about 75,000 daltons (BDH, England), capable of chelating Mn^{++} ions in solution strongly, and polylysine with molecular weight of 4,000, 14,000, 25,000, 55,000, and 75,000 daltons (Sigma, USA), which, to endow it with chelating properties, was treated with EDTA or DTPA by the mixed anhydrides method [7]. To endow the polymers with ability to interact with protein, their free amino groups were modified by treatment with a tenfold molar excess of succinic anhydride in phosphate buffer, pH 8.0, with the polymer concentration 1 mg/ml. The number of free amino groups in the polymer at all stages of the process was determined by titration with trinitrobenzenesulfonic acid [9]. After succinylation, no free amino groups could be determined in the polymers. The polyethyleneimine was succinylated after chelation of Mn, and the polylysine, treated with DTPA or EDTA, could be succinylated either before or after chelation of the corresponding label.

Antibodies or their Fab-fragments were modified by succinylated polymers through water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; from Sigma]. For this purpose the polymer was dissolved in unbuffered medium to a concentration of 1-2 mg/ml; the pH of the solution was 3.5-4.5. If the pH rose above 4.5, it was lowered by addition of HCl. Carbodiimide was then added to the solution up to a concentration of 1-2 mg/ml and the mixture was incubated until a constant pH was established, which was slightly raised (10-15 min). Next, protein was added to the system in the form of a solution in 0.05 M borate buffer, pH 8.2 (protein concentration 1-2 mg/ml), and the resulting solution was incubated for 1 h. To separate the protein–polymer conjugates from free protein and polymer, the reaction mixture was chromatographed on Sephadex G-100, Sepharose 6B, or Ultragel AcA-34 (depending on the molecular weight of the polymer, protein, and conjugate as a whole, the carrier had to enable all three components of the system to be eluted as separated peaks). It was noted that under the conditions chosen the degree of binding of protein with polymer was close to 100%, and virtually no free protein or polymer could be found in the system.

The metal was incorporated into the protein–polymer conjugate by simple incubation of the corresponding salt with the conjugate in physiological saline, pH 7.4, for a few minutes. The free metal was separated by gel-chromatography on Sephadex G-25. The concentration of radioactive metal (^{111}In) was determined with a γ-counter and the Mn and Cd concentrations by NMR spectroscopy, according to values of T_1 and T_2.

Preservation of functional activity of the modified antibodies was determined by solid-phase radioimmunoassay [5].

EXPERIMENTAL RESULTS

As a result of chemical modification of the antibodies and their fragments the following derivatives were obtained (abbreviations used: AB – antibody, Fab – the Fab fragment of the antibody, PEI – polyethyleneimine, PL – polylysine): AB–PEI, AB–$PL_{14,000}$–DTPA, AB–$PL_{25,000}$–DTPA, AB–$PL_{55,000}$–DTPA, AB–$PL_{75,000}$–EDTA, Fab–$PL_{14,000}$–DTPA. Various radioactive (^{111}In) and nonradioactive (Mn, Cd) metals were bound with the resulting derivatives. Incidentally, only Mn bound with AB–PEI, whereas the remaining conjugates were labeled with the various metals depending on the type of experiment.

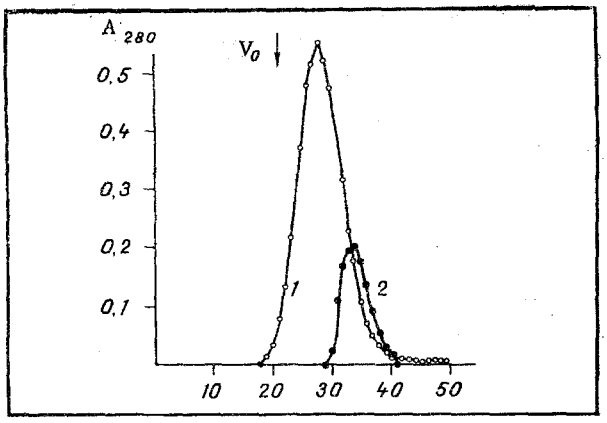

Fig. 1

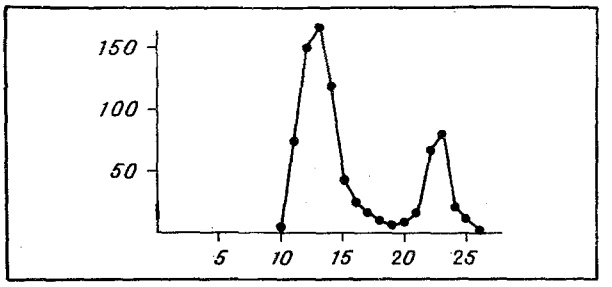

Fig. 2

Fig. 1. Gel filtration of native and modified antibodies on Ultragel AcA-34. Abscissa, nos. of fractions. 1) AB–$PL_{14,000}$–DTPA; 2) AB.

Fig. 2. Gel-filtration of polymer-modified antibodies after binding of $^{111}InCl_3$. Abscissa, numbers of fractions; ordinate, radioactivity of ^{111}In (in μCi). Column 10 ml, Sephadex G-25, volume of fraction 0.5 ml.

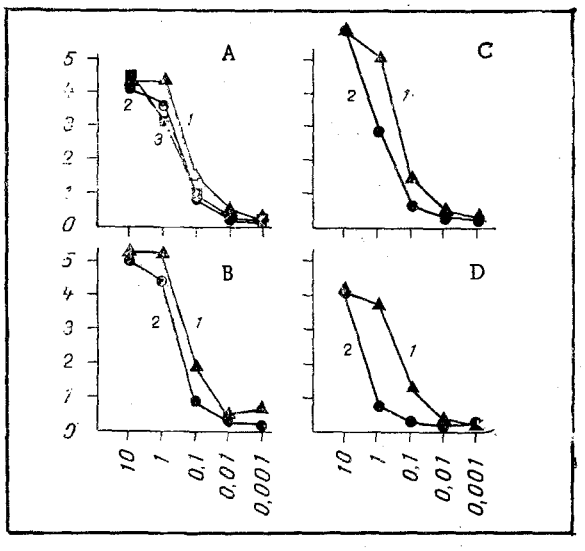

Fig. 3. Solid-phase radioimmunoassay of antibodies and Fab fragments, native and modified by chelating polymers. Abscissa, protein concentration (in μg/mg); ordinate, radioactivity (in cpm × 10^{-3}). A) AB–PEI and B–PEI–MN;B) AB–$PL_{55,000}$–DTPA; C) AB–$PL_{14,000}$–DTPA; D) Fab–$PL_{14,000}$–DTPA. 1) Native protein; 2) protein modified by chelating polymers; 3) protein modified by chelating polymer containing metal.

As a result of modification the molecular weight of the protein was changed; this could be used on one hand, for gel-chromatographic separation of modified from unmodified antibodies, and on the other hand, to estimate the approximate molecular weight of the conjugates in order to determine the quantity of polymer bound with protein. A typical gel-chromatogram is shown in Fig. 1. It was shown by the use of markers that in the case of modifying polymers with molecular weight of 55,000 and 75,000 daltons, the average composition of the conjugate was 1:1, whereas for polymers with molecular weight of 14,000 and 25,000 daltons, one or two polymer chains could be bound with one protein molecule.

Considering that the degree of modification of the polymer by chelating groups (in the case of polylysine) was quite considerable, the modified protein ought to take up a large quantity of metal. The gel-chromatogram of a typical conjugate AB–$PL_{25,000}$–DTPA, after incubation with $^{111}InCl_3$, is shown in Fig. 2. It will be clear from Fig. 2 that more than 70% of the radioactivity was bound with the polymer derivative of the antibody. With the use of a mixture of nonradioactive $InCl_3$ and of fixed amounts of protein, it was calculated that 50-90 In atoms could be bound with one molecule of antibody or of its Fab fragment. The same figures were obtained when the amount of Mn and Cd bound was determined by NMR spectroscopy.

To what extent do the antibodies and their Fab fragments preserve their activity as a result of modification by polymers, and does the presence of chelated metal affect this process? Typical curves for titration of modified antibodies in indirect solid-phase radioimmunoassay using myosin [5] are given in Fig. 3. They demonstrate that activity of the whole antibodies was virtually unchanged as the result of their one- or two-point modification by chelating polymers. The presence or absence of metal had no effect on preservation of activity (data for the remaining preparations are not given because they were exactly the same as those described above). In the case of Fab fragments (Fig. 3), their activity was reduced a little by modification, but as before, it still remained sufficiently high (about 60% of the original value).

It can thus be concluded from the results that, first, modification of antibodies or their fragments by chelating polymers enables as many as several tens of atoms of a heavy metal to be bound to one protein molecule, which is a much higher figure than has hitherto been obtained, and second, that modification has virtually no effect on the specific properties of the antibodies. The possibility of using these conjugates *in vivo* is currently being studied by the present writers.

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IN VIVO PRODUCTION OF MACROPHAGE MIGRATION INHIBITION AND STIMULATION FACTORS DURING THE INDUCTIVE PHASE OF THE ALLOIMMUNE RESPONSE

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The macrophage migration inhibition factor (MIF), one of the most thoroughly studied immunomediators, is usually obtained in culture *in vitro*, where it is produced by immune T lymphocytes, stimulated by a specific antigen, and also by normal lymphocytes, activated by an alloantigen or mitogen [7]. Investigation of the production of MIF and other lymphokines *in vivo* demonstrated activity of these factors in the serum of animals immunized with MCG 4 h after a second intravenous injection of the antigen [11]. The appearance of MIF in the serum or lymph leads to adhesion of the macrophages to the walls of vessels or serous cavities, and to accumulation of cells in regional lymph nodes [10, 12, 16], thereby evidently facilitating cellular cooperation in lymphoid organs.

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