Radioimmunoassay of Bifunctional Monoclonal Antibodies Binding to Antigens

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Along with common monoclonal antibodies hybridoma technology helps obtain bifunctional antibodies carrying binding sites for two different antigens. For this purpose two hybridomata forming different monoclonal antibodies are usually fused together. As a result of recombination of the light and heavy chains of parent Ig in hybrid hybridomata (quadromata), monospecific antibodies of both parent types are formed, bifunctional antibodies are inactive Ig [4]. During the preparation and use of bifunctional antibodies, the problem of their retaining the parent antibody properties arises.

The present research was aimed at solving this problem using bifunctional antibodies specific to α-endorphin (α-END) and horseradish peroxidase (HP) obtained previously by fusing two murine hybridomata, anti-α-END $F_8/4OE_9$ [2,3]. For this purpose α-endorphine binding to bifunctional antibodies formed by quadroma cells and monoclonal antibody binding to α-endorphin obtained from parent hybridoma $F_8/4OE_9$ were studied by radioimmunoassay.

MATERIALS AND METHODS

Cells were cultured in DMEM medium with 10% fetal calf serum (Flow). Bifunctional antibodies were isolated from culture medium or from quadroma clone ascites [2] by consecutive affinity column chromatography with α-endorphin and horseradish peroxidase Sepharose [3].

Parent hybridoma $F_8/4OE_9$ [1] was used as pure monoclonal antibodies to α-endorphin, or monoclonal antibodies were isolated from ascites by chromatography on α-END-Sepharose. Antibodies of both parent murine hybridomata were referred to IgG (IgG2a and Ig1 [2,3]. Antibody levels in eluates and culture medium were measured by radioimmunoassay (RIA). For this purpose affine-purified monoclonal antibodies to α-endorphin and bifunctional antibodies were iodinated by the chloramine method [6] and purified from free ^{125}I by gel filtration on Sephadex G-10. Rabbit antiserum to murine IgG was used in RIA. The incubation medium (total volume 1 ml) during measurements of parent monoclonal antibodies to a-endorphin included 0.1 ml of ^{125}I-labeled monoclonal antibodies to α-endorphin ($13\text{-}115\times10^3$ cpm), during measurements of bifunctional antibodies o.1 ml of ^{125}I-labeled monoclonal antibodies ($13\text{-}115\times10^3$ cpm) = 0.1 ml rabbit antiserum to murine IgG in RIA dilution, 0.1 ml of the test sample of standard antibodies (monoclonal or bifunctional antibodies in concentrations of 1, 2, 4, 8, 16, 32, 64, and 128 ng/sample), and 0.7 ml RIA buffer (0.05 M Na phosphate buffer with 0.15 M NaCl, 0.2% bovine serum albumin, 0.05% Tween-20, and 0.002% NaN_3). Standard antibody solutions were

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made from antibody preparations obtained from ascitic fluid, assuming that A_{280}=1.35 corresponded to a concentration of 1 mg/ml. The samples were incubated at ambient temperature for 16-24 h, after which 0.1 ml donkey antirabbit antibodies diluted 1:5 preincubated with 5% neutral murine serum (1 h at 37°C) and 0.1 ml neutral rabbit serum were added to each tube. Twenty-four hours later, 1.5 ml normal saline was added to the samples, which were then centrifuged for 30 min at 1000 g and 0°C and, after the supernatant s were removed, sediment radioactivity was measured. The control growth medium (DMEM with 10% fetal calf serum) did not influence the binding of labeled bifunctional and monoclonal antibodies to antimurine serum.

The incubation medium for the study of ^{125}I-labeled-α-endorphin binding to monoclonal antibodies to α-endorphin and to bifunctional antibodies consisted of 0.1 ml ^{125}I-α-endorphin (13-15×10^3 cpm), 0.1 ml monoclonal or bifunctional antibodies in concentrations of 1.25, 2.5, 5, 10, 20, 40, and 80 mg/sample, and 0.8 ml RIA buffer, final volume 1 ml. The samples were incubated for 16-24 h at 2°C. Free and bound ^{125}I-α-endorphin were separated as described previously [1]. Radioimmunoassay of α-endorphin using monoclonal antibodies to α-endorphin and bifunctional antibodies with ^{125}I-α-endorphin was carried out as described previously [1]. Microsoft Beckman Immunofit EIA/RIA Analysis (Version 1.00) software was used to plot calibration curves and to assess α-endorphin inhibitory concentrations.

RESULTS

Previously we obtained hybridoma $F_8/4OE_9$ to α-endorphin, an opioid peptide consisting of 17 amino acids [1]. These cells were fused with hybridoma $36F_9$ specific to horseradish peroxidase to form quadromata [2]. About 30% of the immunologically active antibodies formed by these quadromata are bifunctional antibodies specific to α-endorphin and horseradish peroxidase [3]. The present study is devoted to ^{125}I-α-endorphin binding by parent antibodies ($F_8/4OE_9$) and by bifunctional antibodies ($F_8/4OE_9$336F_9); in addition, α-endorphin radioimmune calibration curves obtained with parent monoclonal antibodies to α-endorphin and with bifunctional antibodies were analyzed.

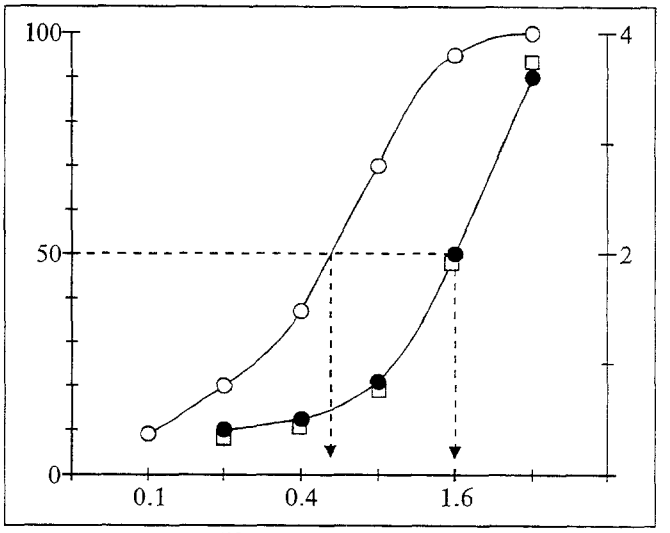

Fig. 1. binding of ^{125}I−α−endorphin to anti−α−endorphin antibodies (*1*), bifunctional antibodies (*2*), and bifunctional antibodies in the presence of 10 μg/sample of horseradish peroxidase (*3*). The arrows show the antibody concentration corresponding to binding equal to half of the maximal.

In the first series of experiments (Table 1, Fig. 1) ^{125}I-α-endorphin binding to monoclonal and bifunctional antibodies was examined. The measurements were carried out with affinity-purified bifunctional antibodies isolated from three samples of culture medium from three quadroma cell clones. The content of bifunctional antibodies in medium samples varied from 35.0 to 87.8 mg/ml, as shown by the results of eluate radioimmunoassay. Two samples of parent hybridoma $F_8/4OE_9$ medium were taken as pure monoclonal antibodies, these samples containing, according to RIA results, 54.0 and 63.8 mg/ml of monoclonal antibodies. Individual measurements were carried out with each sample of monoclonal or bifunctional antibodies; the antibody concentration providing ^{125}I-α-endorphin binding equal to half of the maximal binding was taken as the parameter characterizing antibody properties (Fig. 1). In our experiments (the groups of independent experiments differed in terms of ^{125}I-α-endorphin lots) the antibody concentration ensuring 50% binding of ^{125}I-α-endorphin was 0.93±0.17 and 0.57±0.10 μg/sample for monoclonal antibodies and 2.76±0.29 and 2.0±0.48 μg/sample for bifunctional antibodies.

TABLE 1. Antibody Concentrations Corresponding to 50% Binding of Immunoreactive ^{125}I−α−Endorphin, mg/sample (*M*±*m*, Number of Measurements Indicated in Parentheses)

Antibodies	Experiment № 1	Experiment № 2
Anti−α−endorphin $F_8/4OE_9$	0.93±0.17 (2)	0.57±0.10 (2)
Bifunctional $F_8/4OE_9$×36F_9	2.76±0.29 (3)	2.02±0.48 (3)
Bifunctional in the presence of horseradish peroxidase, 10 μg/sample	–	2.32±0.33 (3)

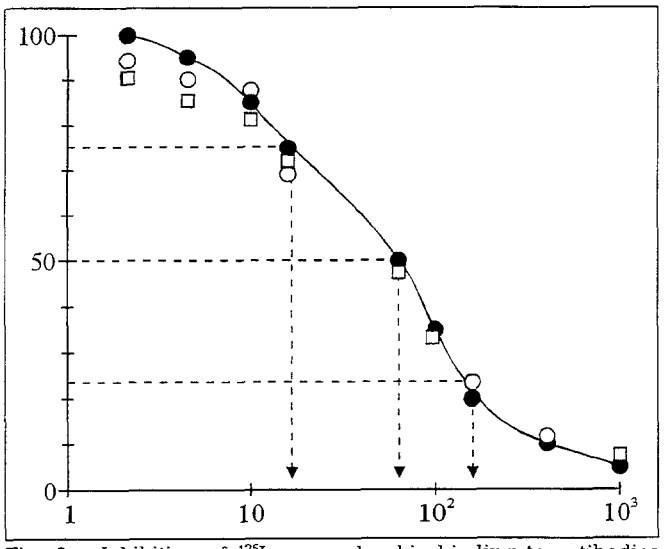

Fig. 2. Inhibition of ^{125}I–α–endorphin binding to antibodies to α–endorphin by unlabeled α–endorphin. *1*) monoclonal antibodies to α–endorphin; *2*) bifunctional antibodies (α–endorphin/horseradish peroxidase); *3*) bifunctional antibodies in the presence of 10 μg/sample of horseradish peroxidase. B_0 – ^{125}I–α–endorphin binding to antibodies in the presence of α–endorphin; B – the same in the presence of α–endorphin. The arrows show unlabeled α–endorphin concentrations inhibiting binding of ^{125}I–α–endorphin to antibodies by 25% (left), 50% (central), and 75% (right arrow).

Hence, 3 to 3.5 times higher volumes of bifunctional antibodies were needed to bind the same ^{125}I-α-endorphin quantities in comparison with monoclonal antibodies. Theoretically, double volumes of bifunctional antibodies are needed as against parent antibodies, provided that the individual antigen-binding centers of bifunctional antibodies retain the parent antibody affinity. Deviation from the theoretically expected value may be due not to affinity reduction but to loss of a part of the activity of bifunctional antibodies after two affinity chromatographic sessions under stringent conditions (pH 2.2), whereas monoclonal antibodies were not exposed to this procedure in this series of experiments. An identical pattern of curves reflecting labeled ligand binding to monoclonal and bifunctional antibodies (Fig. 1) is probably indicative of an unchanged affinity of bifunctional antibodies as against parent monoclonal antibodies.

It was of special interest to elucidate whether bifunctional antibody antiendorphin shoulder binding to α-endorphin depended on the other shoulder (whether it were free or not) or was related to its own horseradish peroxidase antigen. To answer this question we compared ^{125}I-α-endorphin binding with bifunctional antibodies in the absence of horseradish peroxidase and in the presence of its excess (10 μg/ml). No reliable differences in the parameter characterizing the binding were detected in the presence of horseradish peroxidase excess (Table 1, Fig. 1).

Radioimmunoassay of α-endorphin with monoclonal and bifunctional antibodies were compared. The same antibody lots were used as in previous experiments. One can see on Fig. 2 virtually identical calibration curves reflecting experiments with monoclonal and bifunctional antibodies both in curve parameters (α-endorphin concentrations inhibiting ^{125}I-α-endorphin binding with monoclonal and bifunctional antibodies by 25, 50, and 75%) were unreliable (Table 2). We consider that our results indicate a similar affinity of the antiendorphine shoulder of bifunctional antibodies and of parent monoclonal antibodies with respect to α-endorphin. Furthermore, our data demonstrate that horseradish peroxidase antigen does not influence bifunctional antibody binding to one of the antigens, a-endorphin.

Hence, our results, like other data [5], show that individual antigen-binding sites of bifunctional antibodies appear to retain parent antibody affinity, a fact which is important for the use of these antibodies in immunohistochemistry and enzyme immunoassay and as possible transporters of physiologically active substances to target cells.

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TABLE 2. ^{125}I–α–Endorphin Binding to Antibodies to α–Endorphin and Bifunctional Antibodies by Unlabeled α–Endorphin ($M\pm m$, $n=3$)

Antibodies	α-Endorphin concentration, nM		
	25% inhibition	50% inhibition	75% inhibition
Anti–α–endorphin $F_8/4OE_9$	62.9±0.4 (2)	185±12 (2)	524±53 (2)
Bifunctional $F_8/4OE_9\times36F_9$	42.6=6.7	133±15	408±22
Bifunctional in the presence of horseradish peroxidase, 10 μg/sample	41.1±6.5	120±13	383±16