Use of a Sensitive Bioimmunoabsorbent Assay to Isolate and characterize Monoclonal Antibodies to Biologically Active Human Erythropoietin

By Albertus W. Wognum, Peter M. Lansdorp, Connie J. Eaves, and Gerald Krystal

At present, one of the most sensitive assays for human erythropoietin (Ep) is a bioassay that measures the Ep-dependent proliferation of spleen cells from phenylhydrazine-treated mice after 24 hours in culture. We describe how this assay can be used as the basis of a very sensitive method for detecting mouse antibodies to biologically active human Ep. In this procedure, microtiter wells are first coated with goat anti-mouse Ig antibody, then treated with mouse antibodies (serum or hybridoma culture supernatants), and finally incubated with a fixed amount of pure human Ep. Specific binding of anti-Ep antibodies is detected by adding spleen cells from phenylhydrazine-treated mice to the wells and measuring the ability of the cells to incorporate 3H-thymidine 24 hours later. This bioimmunoabsorbent assay (BISA) revealed the presence of anti-Ep antibodies in sera from mice immunized with either pure human urinary Ep or a synthetic dodecapeptide corresponding to the aminoterminal region of Ep and in the culture supernatants from three of eight stable anti-Ep antibody-producing hybridoma cell lines that we have isolated. The three monoclonal antibodies showed similar reactivities in the BISA, but showed different affinities for Ep, with Kd values of ~0.7, 8, and 240 nmol/L, respectively. Further studies showed that all antibodies were capable of neutralizing Ep bioactivity and of binding 125I-labeled Ep in a radioimmunoabsorbent assay (RIA) but were virtually unreactive to Ep adsorbed to the bottom of enzyme-linked immunoabsorbent assay (ELISA) wells. Our results suggest that the BISA strategy may be an important complement to conventional RIA and ELISA techniques for identification of monoclonal antibodies specific for biologically active growth factors.

THE GLYCOPROTEIN hormone erythropoietin (Ep) has an important and well-established role in regulating the development of RBCs from committed erythroid precursors. Although pure hormone was first isolated in 1977 and the gene for Ep was cloned and expressed in 1985, progress concerning its molecular and functional characteristics has been slow. This may, in part, be due to difficulties that have been widely encountered in the isolation of monoclonal antibodies to biologically active Ep. To date, very few anti-Ep monoclonal antibodies have been described and these have proven of limited value because of their low affinity for the hormone.

Key to the successful isolation of monoclonal antibodies is the specificity, sensitivity, and ease of the screening procedure used. Screening with enzyme-linked immunoabsorbent assay (ELISAs) in which Ep is noncovalently bound to a solid-phase is dependent on the purity of the antigen upon binding to a solid-phase and may result in the disappearance of antigenic sites. For similar reasons, radioimmunoassays (RIAs) in which 125I-labeled Ep is used may also not be ideal. Radiolabeling of Ep results in structural modifications that may not lead only to inactivation of biologic activity but also to changes in immunologic properties. In addition, the specificity of both the ELISA and RIA is dependent on the purity of the antigen preparation since, with these assays, antibodies against impurities cannot be distinguished from antibodies specific for Ep.

These problems can be avoided by using a screening strategy that uses unlabeled, native hormone to detect antigen/antibody binding through the hormone’s biologic activity. In this report, we describe such an assay for the detection of antibodies to Ep. Antibody-containing preparations are immobilized and then detected by their ability to bind and subsequently release picogram amounts of biologically active Ep, which in turn stimulates the in vitro proliferation of spleen cells from phenylhydrazine-treated mice. Using polyclonal murine antiserum raised against both human urinary Ep and a synthetic dodecapeptide corresponding to the aminoterminal region of Ep, we have found that this bioimmunoabsorbent assay (BISA) is highly sensitive and specific for anti-Ep antibodies. Subsequent use of this assay together with an RIA, ELISA, and a neutralizing antibody assay to screen hybridomas has resulted in the detection and isolation of eight monoclonal antibodies to human urinary Ep. One of the three that are reactive in the BISA has a high affinity for the biologically active native hormone.

MATERIALS AND METHODS

Antigen preparation and immunization. Ep was purified to homogeneity from the urine of aplastic anemia patients as previously described and used for all immunizations. Adult C3H/He and C3H/He × C57B1/6J (B6C3F1) mice were immunized by intraperitoneal (IP) injections of 500 U Ep (250 U Ep in complete Freund’s adjuvant (CFA) plus 250 U Ep in non-sodium dodecyl sulfate (SDS) polyacrylamide gel). Mice that developed a strong anti-Ep response (as detected by RIA; see below) after three to four

From Terry Fox Laboratory, B.C. Cancer Research Centre, Vancouver, B.C., Canada; and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, incorporating the Laboratory of Experimental and Clinical Immunology of the University of Amsterdam.

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Address reprint requests to G. Krystal, PhD, Terry Fox Laboratory, B.C. Cancer Research Centre, 601 West 10th Ave, Vancouver, B.C. V5Z 1L3.

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immunizations were then given 500 U Ep intravenously (IV), and the immune spleen cells were used for hybridoma production 4 days later. The 13-amino acid synthetic peptide representing the 12 aminoterminal amino acid residues of human Ep plus an extra cysteine residue at the carboxyterminus was coupled to the carrier proteins keyhole limpet hemocyanin (KLH) and chicken gamma globulin (CGG) using 3-maleimidobenzoic acid-N-hydroxy-succinimide ester (MBS). The 10-amino acid synthetic peptide representing the 10 carboxyterminal residues of human Ep was coupled to KLH and CGG using glutaraldehyde. The synthetic peptides were gifts from Dr H. Ploegh (Netherlands Cancer Institute, Amsterdam) and Dr J. Haaijman (Medical Biological Laboratory, TNO, Rijswijk). Balb/c mice were immunized IP with 50 to 100 μg peptide-carrier conjugates emulsified in CFA on three occasions at 3-week intervals. Blood samples were obtained 3 weeks after the third immunization and tested for the presence of antipeptide and anti-Ep antibodies. Mice showing a detectable anti-Ep response received a fourth immunization and were used 4 days later for hybridoma production.

Hybridoma production. Fusions between immune spleen cells and SP 2/0 or NS1 myeloma cells were performed essentially as described by Fazekas de St Groth and Scheidegger. Hybridoma culture supernatants were harvested 12 to 15 days after the fusion and tested for anti-Ep antibodies. Positive hybridomas were isolated by double cloning in 0.8% methyl cellulose medium or by limiting dilution in suspension cultures in the presence of either spleen feeder cells or 1% human monocyte conditioned medium as a source of hybridoma growth factor (HGF).

Antibody production, purification, and quantitation. Preparative amounts of monoclonal antibodies were obtained by ascites production in mice and by growing hybridomas in roller bottle cultures at low concentrations of fetal calf serum (FCS). IgG, monoclonal antibodies were purified from ascites or concentrated hybridoma culture supernatants by ammonium sulfate precipitation at 45% saturation followed by cation-exchange chromatography over fast-flow S-Sepharose (Pharmacia, Uppsala, Sweden) as described by Boot et al. IgG monoclonal antibodies were partially purified from concentrated culture supernatants by size-exclusion chromatography over a 2.6 x 85-cm Sephadex G200 (Pharmacia) column equilibrated with 0.65 mol/L sodium chloride in 20 mmol/L sodium phosphate buffer, pH 7.4. Further purification was achieved by anion-exchange chromatography. In brief, the IgG-containing fractions eluted from the G200-column were pooled, concentrated by ultrafiltration over YM10 filters (Amicon, Danvers, MA), dialyzed against 20 mmol/L Tris buffer, pH 8, and loaded onto a 1-mL column of fast-flow Q-Sepharose equilibrated with the same buffer. The column was eluted with a linear gradient of 0 to 500 mmol/L sodium chloride in Tris buffer at a flow rate of 0.4 mL/min, and 1-mL fractions were collected. Antibody concentrations were measured in a competition RIA as described by Boot et al. Mouse Ig subclasses were determined by ELISA using rat antibodies specific for murine heavy and light chain isotypes.

BISA. For detection of antibodies to native unlabelled Ep, 96-well immunoplates (Nunc, Copenhagen) were coated with affinity-purified goat antibodies against mouse Ig. Mouse sera or hybridoma culture supernatants diluted in PBS + 1% BSA were incubated in these wells for 4 hours at 37°C, followed by incubation overnight at room temperature with pure human urinary Ep (80,000 U/mg) labeled with 121I using the chloramine-T method. The specific activity of radiolabeled Ep ranged from 900 to 2,700 Cu/mmol (0.4 to 1.25 mol 121I/mol Ep). After washing with PBS, bound radioactivity was eluted from the wells with 100 μL 2 mol/L NaOH, transferred to polystyrene tubes, and counted in a γ counter.

Measurement of dissociation constants. Equilibrium binding of 121I-Ep to anti-Ep antibodies was performed in radioimmunoprecipitation experiments. Various antibody concentrations (0 to 0.5 μmol/L) were incubated with a fixed amount of 121I-Ep (50,000 cpm) in 50 μL PBS + 1% BSA in 0.5-mL microfuge tubes. After 16-hour incubation at room temperature, 50 μL 20% (vol/vol) dilution of goat anti-mouse Ig antiserum in PBS + 1% BSA and 10 μL 1% suspension of formalin-fixed Staphylococcus aureus cells (Pansorbin, Calbiochem, LaJolla, CA) were added to each sample to
monoclonal antibodies to erythropoietin

fig 1. BISA reactivity of immune sera: microtiter wells, previously coated with goat antibodies to mouse Ig were first incubated with the indicated dilutions of sera from mice immunized with human urinary Ep (B), the KLH-conjugated aminoterminal synthetic peptide (A) or the KLH conjugated carboxyterminal synthetic peptide (A). and then incubated overnight with human urinary Ep (1 U/well). The activity of the Ep bound was determined by measuring 3H-thymidine incorporation 24 hours after the addition of spleen cells from phenylhydrazine-treated mice (400,000/well). 3H-thymidine incorporation in wells incubated without mouse antibodies (X). Each point represents the mean of triplicate samples; SE, vertical bars. No bars are shown in instances in which the SE was covered by the graphical representation of the mean.

precipitate mouse antibodies and immune complexes. Precipitates were collected after one to four hours by centrifugation and counted in a radioactivity counter. The equilibrium dissociation constant (Kd) was calculated by determining the concentration of antibody that bound 50% of the maximum amount of bindable 125I-Ep, as calculated from the reciprocal value of the ordinate intercept in plots of 1/(amount of bound) vs 1/(amount of antibody added), as described by Calvo et al. Alternatively, radioimmunoprecipitation experiments were performed at constant antibody concentrations (100 pmol/L) and variable amounts of 125I-Ep (15,000 to 300,000 cpm). In this instance, Kd values were obtained by Scatchard analysis.

results

BISA titration of anti-Ep activity in immune sera. To identify antibodies to biologically active Ep, we developed an assay in which Ep is first bound to solid-phase antibodies. Because of the sensitivity of existing bioassays, we then tested for the release of sufficient Ep to stimulate detectable levels of proliferation of spleen cells from phenylhydrazine-treated mice as described in the Materials and Methods Section. Figure 1 shows an example of the strong BISA reactivity exhibited by a serum from a mouse immunized with pure (80,000 U/mg) human urinary Ep. The BISA titer of this serum was 1:500,000 (P < .001, one-tailed t test). With sera raised against irrelevant antigens, 3H-thymidine incorporation was never found to exceed background levels at any dilution tested. Strong to moderate Ep-binding activity was detected in sera from 10 of 12 mice immunized with Ep. The other two, immunized according to the same protocol, never showed a detectable anti-Ep response using the BISA assay (data not shown).

The BISA assay also detected weak but significant anti-Ep activity in the sera of two of ten mice immunized with a synthetic peptide representing the 12 aminoterminal amino acid residues of Ep. As shown in Fig 2 for one of these sera, the binding of Ep was selectively inhibited in a dose-dependent manner by unlabeled or carrier-conjugated aminoterminal peptide but not by a synthetic peptide representing the 10 carboxyterminal amino acid residues of Ep. These results confirm previous reports that some antibodies generated against epitopes of the aminoterminal region of Ep cross-react with native human Ep. In contrast, no Ep-binding activity was detectable in the sera from 10 of 10 mice immunized with the carboxyterminal Ep peptide (Fig 1), even though all 10 of these sera had a high titer (> 1:1,000) in an ELISA using the carboxyterminal peptide as antigen (data not shown). This latter result suggests that antibodies directed against the carboxyterminal peptide may not share cross-reactivity with the native Ep molecule.

isolation of monoclonal antibodies to Ep. Attempts to isolate hybridomas producing monoclonal antibodies to Ep from any of the mice immunized with synthetic peptides were unsuccessful. Several hybridomas that produced antibodies specific for the aminoterminal peptide were obtained from mice immune to this antigen, but none of these proved reactive with native human Ep (data not shown). Fusion experiments with spleen cells from eight mice immunized with Ep yielded eight stable hybridoma cell lines that produced anti-Ep antibodies as detected by at least one of the four assays used, ie, BISA, neutralization, RIA, or ELISA.

fig 2 competitive inhibition of Ep binding to antipeptide antibodies by the aminoterminal dodacapeptide as detected in the BISA. Binding of native Ep to antibodies raised against the KLH conjugated dodacapeptide (B), or against a control antigen (C) was tested with increasing concentrations of unconjugated dodacapeptide (peptide 1-12; A), the same peptide conjugated to CGG (B), or the carboxyterminal peptide (peptide 157-166; C). Each point is the mean 3H-thymidine incorporation of triplicate samples. SE was similar to those shown in legend to Fig 1.
Supernatants of these clones were clearly produced antibodies that were reactive in the ELISA. The ability of monoclonals was only weakly reactive in the RIA, neutralization assay, or RIA, with antibody 26 showing a stronger reactivity to \(^{125}\)I-Ep than antibody 16. Antibody 2 was only weakly reactive in the RIA (Fig 4). This is most likely caused by a low affinity of antibody 2 for Ep (discussed below).

The anti-Ep monoclonals were also tested in an ELISA for their reactivity with pure human Ep noncovalently absorbed to plastic microtiter wells. Although anti-Ep immune sera showed a strong reactivity in the ELISA, binding of Ep by antibodies 2 and 16 could not be detected in this assay (Table 1). Antibody 26 showed some reactivity but only at concentrations >2.5 \(\mu\)g/mL, indicating a >100-fold lower reactivity in this assay than in the BISA, neutralization assay, or RIA (data not shown). The virtual absence of reactivity of the antibodies in the ELISA and the weak reactivity of antibody 2 in the RIA illustrate the disadvantages that these latter assays may have as initial screening procedures to detect anti-Ep-producing hybridomas. This was further supported by additional comparisons of the reactivities of the five other monoclonal anti-Ep antibodies isolated that were negative in the BISA assay (Table 1). Two of these five were reactive in the ELISA but not in the RIA, one was detectable in the RIA but not in the ELISA, and only two could be detected in both assays (Table 1). None of the five was capable of neutralizing the biologic activity of Ep. Thus, all five antibodies that were negative in the ELISA assay appear to be specific for antigenic determinants present only when Ep is immobilized on a solid phase or labeled with \(^{125}\)I and not found on native Ep in solution.

Neutralizing activity of the anti-Ep antibodies detectable by BISA. As shown in Fig 5, the biologic activity of 50 mU/mL Ep could be inhibited by antibodies 16 and 26. These antibodies significantly inhibited the \(^{3}\)H-thymidine incorporation of the Ep-dependent target cells at concentrations as low as 5 to 10 ng/mL (\(P < .01\), one-tailed \(t\) test). At antibody concentrations >2.5 \(\mu\)g/mL, virtually all of the Ep activity was neutralized. Antibody 2 also demonstrated neutralizing activity in the same type of assay but only at concentrations >0.3 \(\mu\)g/mL (\(P < .05\), one-tailed \(t\) test). No inhibitory activity was detected in this assay with an antibody of irrelevant specificity (Fig 5), and none of the anti-Ep monoclonals were able to inhibit the \(^{3}\)H-thymidine incorporation of an IL-2-dependent CTLL cells at any concentration tested up to 5 \(\mu\)g/mL (data not shown). These results provide strong evidence for the specificity of the anti-Ep neutralizing activity of the monoclonals isolated in the present study. They also suggest that the Ep bioactivity detected in the BISA does not result from Ep bound to the...
immobilized antibodies, since, in this bound form, the activity of Ep is neutralized. To examine the fate of antibody-bound Ep during the biologic part of the BISA, time-course studies using labeled Ep as a tracer were performed. As shown in Table 2, substantial amounts of $^{125}$I-Ep bound to the immobilized antibodies were released within 24 hours after addition of Ep-dependent target cells to the assay wells. A similar release of $^{125}$I-Ep, although at a slower rate, was observed after the addition of medium alone (data not shown). Release of $^{125}$I-Ep was particularly rapid for antibody 2, with half of the $^{125}$I-Ep released within minutes after addition of the target cells and was much slower for the Ep bound to immobilized antibodies 16 and 26. One-half of the $^{125}$I-Ep originally bound to antibody 16 was released between two and five hours of culture and one-half of the $^{125}$I-Ep bound to antibody 26 was released after ~11 hours. Because even late addition of some Ep can give a detectable response in a similar assay, slow release of Ep even by strongly neutralizing antibodies could be expected to allow their detection in the BISA.

Affinity constants of monoclonal anti-Ep antibodies 2, 16, and 26. The dependency of BISA activity on the release of bioactive Ep might suggest that this assay would preferentially detect low-affinity neutralizing anti-Ep antibodies. To measure the dissociation constants of the anti-Ep antibodies isolated in this study, we used a radioimmunoprecipitation procedure. Increasing concentrations of a given antibody were incubated with a fixed amount of $^{125}$I-Ep. Plots of $1/\text{radioactivity (cpm) bound} \times 1/\text{concentration of anti-Ep added}$ are shown for antibody 2 (Fig 6A), antibody 16 (Fig 6B), and antibody 26 (Fig 6C). The maximum amount of $^{125}$I-Ep that could bind to each antibody was calculated from the reciprocal of the ordinate intercept in each plot, and these values (17,000 cpm, 16,200 cpm, and 21,600 cpm for antibodies 2, 16, and 26, respectively) were used to calculate the $K_a$ for each antibody from the antibody concentration required to bind 50% of the maximal amount of bindable $^{125}$I-Ep. As shown in Table 3, a relatively high $K_a$ was determined for antibody 2, indicating that this antibody has a low affinity for Ep. However, much lower $K_a$ values were measured for antibodies 16 and 26, indicating that these antibodies have a moderate and high affinity for Ep, respectively. For antibody 26, the $K_a$ was also determined by

![Figure 5](image5.png)

**Fig 5.** Effect of monoclonal antibodies on the in vitro biologic activity of Ep: 5 mU human urinary Ep in 50 µL culture medium was preincubated with the indicated concentrations of anti-Ep or control monoclonal antibodies, after which Ep bioactivity was determined as described in the Materials and Methods Section.

**Table 2.** Release of $^{125}$I-Labeled Ep in the BISA

<table>
<thead>
<tr>
<th>Incubation Time* (h)</th>
<th>Release Ep (%)</th>
<th>Anti-Ep 2</th>
<th>Anti-Ep 16</th>
<th>Anti-Ep 26</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>47 ± 5</td>
<td>35 ± 1</td>
<td>29 ± 1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>75 ± 8</td>
<td>42 ± 1</td>
<td>31 ± 1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>86 ± 4</td>
<td>46 ± 2</td>
<td>35 ± 1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>89 ± 1</td>
<td>56 ± 1</td>
<td>41 ± 1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>90 ± 2</td>
<td>54 ± 1</td>
<td>44 ± 9</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>93 ± 4</td>
<td>55 ± 2</td>
<td>49 ± 1</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>92 ± 3</td>
<td>58 ± 2</td>
<td>61 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

Microtiter wells were coated with anti-mouse Ig and incubated with anti-Ep monoclonal antibodies 2, 16, and 26 (0.5 µg/mL) and subsequently with Ep. Wells were washed and incubated at 37 °C with spleen cells from phenylhydrazine-treated mice for the indicated times, after which the amount of radioactivity present in the culture supernatant was measured.

*Expressed as percentage of the $^{125}$I-Ep originally bound in the wells, i.e., 4,870 ± 70 cpm, 19,310 ± 170 cpm, and 33,280 ± 220 cpm for antibodies 2, 16, and 26, respectively. Data are the means ± SEM of triplicate samples.

![Figure 6](image6.png)

**Fig 6.** Radioimmunoprecipitation of $^{125}$I-Ep by anti-Ep monoclonal antibodies. Various concentrations of antibody 2 (A), antibody 16 (B), and antibody 26 (C) were equilibrated with a fixed amount of $^{125}$I-Ep (70,000 cpm). The radioactivity precipitated with anti-mouse Ig antiserum and Staphylococcus aureus cells was then counted and $1/\text{radioactivity (cpm) specifically bound}$ was plotted vs $1/\text{concentration of antibody added}$. Correlation coefficients were 0.989 (A), 0.996 (B), and 0.994 (C).
Scatchard analysis in experiments in which a constant concentration of anti-Ep was equilibrated with various amounts of \( ^{125}\text{I}-\text{Ep} \) (Table 3). The value obtained, 0.39 nmol/L, is in good agreement with the \( K_d \) value of \( \approx 0.7 \) nmol/L obtained in the reciprocal experiment and confirmed that antibody 26 has a high affinity for Ep. These results clearly establish that monoclonal antibodies isolated with the BISA can have low and moderate as well as high affinities for Ep.

**DISCUSSION**

In this report, we describe a new BISA that is useful for detection of monoclonal antibodies reactive with native, biologically active, human Ep. The BISA is a combination of a solid-phase immunoassay and a standard in vitro bioassay for Ep, performed sequentially in the same microtiter wells. The major advantage of the BISA is that binding of Ep to antibodies is detected by the functional activity of the antigen itself. Consequently, only antibodies specific for native, biologically active Ep are detected. This property makes the BISA more suitable in screening for monoclonal antibodies to native Ep than perhaps simpler and faster assays, such as the RIA and ELISA. Our findings, as presented in Table 1, demonstrate the limitations of techniques in which structurally or conformationally changed antigen molecules are used for isolation of monoclonal antibodies to soluble antigens such as Ep. Moreover, in the BISA, impure hormone preparations can be used as antigen, since the in vitro proliferation assay is relatively specific for Ep. As shown previously by our group and by other researchers, agents other than Ep do have some effects on \(^3\text{H}\)-thymidine incorporation, but the magnitude of these nonspecific effects is very small as compared with the response to human Ep.

On first consideration, it might appear that an assay in which bound Ep is measured by its biologic activity would be restricted to the detection of non-neutralizing antibodies or to neutralizing antibodies of low affinity. However, this proved not to be the case. All three monoclonal anti-Ep antibodies positive in the BISA assay were also capable of neutralizing Ep bioactivity, and two of these antibodies have a moderate to high affinity for Ep. The explanation for this apparent discrepancy lies in the fact that immune complex dissociation occurs and some of the originally bound Ep is released into the culture medium. The reactivity of neutralizing antibodies in the BISA is thus dependent on the amount of bound Ep that is subsequently released in a biologically active form. This amount is determined by the affinity of the antibodies in two ways, as was suggested by the results of the tracer release experiments presented in Table 2. Low-affinity antibodies, like antibody 2, release bound Ep at a relatively high rate, but also bind a relatively small amount of Ep initially, as compared with high-affinity antibodies, like antibodies 16 and 26. These latter antibodies release Ep at a lower rate but can still produce a sufficiently strong signal in the BISA because the amount of Ep bound initially is higher than the amount bound by low-affinity antibodies. Such a mechanism would explain why the BISA reaction patterns of the three antibodies, as presented in Fig 3, are similar despite the large differences in relative affinities to Ep. The differences in affinity of the antibodies for Ep are expressed more clearly in the reaction patterns observed in the neutralization assay, with antibody 2 requiring a much higher concentration than the other antibodies to reach a similar level of inhibition of Ep bioactivity.

The detection with the BISA of antibodies to native human Ep in sera from mice immunized with a 12-amino acid synthetic peptide homologous to the aminoterminal sequence of Ep confirms that this peptide can elicit the production in mice of antibodies that cross-react with human Ep. Similar results were obtained previously by Sue, Sytkowski, and Fisher, who used an aminoterminal 26-residue synthetic peptide to develop both polyclonal antisera to human Ep and a monoclonal anti-Ep antibody. Our inability to detect anti-Ep antibodies in sera from mice immunized with the carboxyterminal decapetide and similar results obtained by Sytkowski and Donahue suggest that this sequence, unlike the aminoterminal peptide, may be inaccessible or has a quite different conformation in the native human Ep molecule.

Using the BISA procedure, we isolated and characterized three new monoclonal antibodies that bind biologically active human Ep. One of these has potent neutralizing activity, which should be of value for further studies of the role of Ep in mediating various cellular responses. One is only weakly neutralizing due to its lower affinity, thus more closely resembling the nonneutralizing antibodies described by Weiss et al and Sytkowski and Fisher. In addition, we isolated five other anti-Ep antibodies that are negative in the BISA and neutralization assays but are positive with RIA and/or ELISA. These thus appear to resemble more closely the anti-Ep antibody described by Yanagawa et al that binds to an epitope on a conformationally altered form of the native Ep molecule. We are now investigating the application of our antibodies in novel immunoassay procedures for measurement of Ep at sensitivity levels comparable to those obtained with current bioassays or with conventional RIA procedures using rabbit anti-Ep sera. In addition, the availability of new series of monoclonal antibodies to Ep of varying specificity should prove valuable for future studies of the structure, conformation, and receptor binding site of the Ep molecule.

**Table 3. Dissociation Constants of Monoclonal Antibodies to Ep**

<table>
<thead>
<tr>
<th>Antibody No.</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
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<tbody>
<tr>
<td>2</td>
<td>241</td>
<td>246</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>7.8</td>
<td>9.7</td>
<td>ND</td>
</tr>
<tr>
<td>26</td>
<td>0.68</td>
<td>0.66</td>
<td>0.39</td>
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\*Radioimmunoprecipitation experiments were performed as described in the Materials and Methods section and in the legend to Fig 6. \( K_d \) values were determined from the antibody concentrations required to precipitate 50% of the maximum amount of precipitable \( ^{125}\text{I}-\text{Ep} \) determined from double reciprocal plots as shown in Fig 6 for experiment 1. The results of a second separate experiment (experiment 2) are also shown.

\( \dagger \) \( K_d \) was obtained by Scatchard analysis of data from binding experiments in which a constant concentration of anti-Ep (100 pmol/L) was equilibrated with various amounts of \( ^{125}\text{I}-\text{Ep} \) (15,000-300,000 cpm).
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