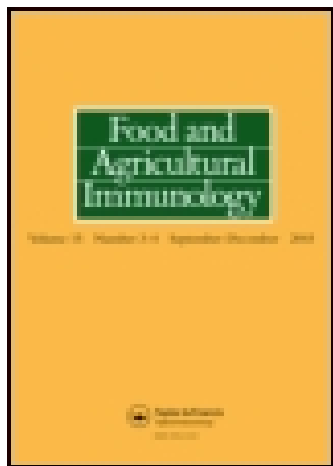




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Monoclonal Antibody Based Two-site Enzyme Immunoassays for Wheat Gluten Proteins. 1. Kinetic Characteristics and Comparison with Other ELISA Formats

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Solid-phase two-site enzyme immunoassays were developed using a number of monoclonal antibodies with differing specificities for wheat grain gluten proteins. While polystyrene microwells performed well in the assay, very high levels of non-specific binding occurred with PVC (polyvinylchloride) microwells in the absence of 'capture' (solid-phase bound) antibody. This was due to binding of complexes of antigen and labelled antibody, and could only be reduced by the use of rather stringent blocking conditions. Assays involving either the simultaneous or sequential addition of antigen and labelled antibody were developed, the latter providing superior results. A number of successful two-site assays were constructed using single monoclonal antibodies, suggesting that repeating epitopes, known to occur in gluten proteins, were commonly recognized. Pairs of monoclonal antibodies with dissimilar antigenic specificities did not function in the two-site assay. A comparison of monoclonal antibody based two-site and antigen-competition immunoassays for gliadin was made. Several antibodies performed considerably better in one format than the other; thus, both assay formats will be of use in quantification of specific gluten protein subclasses.

INTRODUCTION

The two-site or 'sandwich' enzyme-linked immunosorbent assay is the most commonly used solid-phase immunoassay for the quantification of protein antigens. These assays, in which one antibody is bound to a solid support, the antigen-containing sample is added and then the enzyme-labelled antibody, have been extensively developed for both monoclonal and polyclonal antibodies (Voller, 1980). Reasons for the popularity of this assay format include ease of adsorption of antibodies to solid phases such as plastic microwells; the ability to manipulate assay specificity by the use of two

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different antibodies; the sensitivity of the assay format; and the ease of interpretation of results, as increasing antigen concentrations yield increasing ELISA product absorbances (Tijssen, 1985).

Immunochemical methods have been increasingly applied, over recent years, to the study of both the structure and function of cereal grain proteins and the role of gluten proteins in coeliac disease (reviewed in Skerritt, 1988). These considerations had led to attempts to quantify specific gluten fractions by indirect (McKillop *et al.*, 1985; Skerritt, 1985), antigen-competition (Windemann *et al.*, 1982; Troncone *et al.*, 1986; Skerritt *et al.*, 1987) or two-site enzyme immunoassay. Development of sandwich ELISAs for gluten has largely concentrated on analyses of the more soluble protein fraction (gliadin), and the use of one or two polyclonal antisera (Lane *et al.*, 1982; Windemann *et al.*, 1982; Fritschy *et al.*, 1985; McKillop *et al.*, 1985; Troncone *et al.*, 1986; Ayob *et al.*, 1988). These studies have demonstrated that such assays for gliadin are feasible, but a detailed analysis of specificity characteristics and kinetics has not been performed. Recently, Freedman *et al.* (1987) and Mills *et al.* (1989) described a two-site enzyme immunoassay using polyclonal 'capture' (solid-phase bound) antibodies and unlabelled mouse monoclonal antibodies (MAb) in the solution phase. MAb binding was detected by addition of enzyme-labelled anti-mouse antibody.

In this paper, we describe the development of MAb-based two-site assays for wheat gluten proteins using a large panel of labelled and unlabelled antibodies having a range of specificity types. The effects of solid-phase and incubation conditions are examined, and a comparison made with antigen-competition assays performed with several of the same antibodies. In the following paper, the specificity characteristics of a number of combinations of these monoclonal antibodies for different gliadin and glutenin fractions and storage proteins from other cereals is examined. By studies of the inhibition of binding of labelled MAb by unlabelled MAb, antibody specificity and epitope relationships were related to the function of different antibody pairs in the two-site assays.

MATERIALS AND METHODS

Monoclonal Antibodies and Antigens

The specificities of individual monoclonal antibodies used in this study (and the accompanying paper, Skerritt *et al.*, 1989) have been determined using several electrophoresis/immunoblotting and other ELISA systems (Skerritt & Underwood, 1986; Skerritt, 1988; Donovan *et al.*, 1989; Skerritt & Robson, 1990), and are described in terms of the major classes of wheat grain storage proteins. These proteins are: (i) gliadins (lacking intermolecular disulphide bonds, *M_r* 30 000–70 000), subdivided into α -, β -, γ - and ω -gliadins, on the basis of decreasing mobility at acidic pH; and (ii) glutenins (having intermolecular disulphide bonds); subunits in order of decreasing molecular weight are termed (A- (high molecular weight, HMW)), and B- and C- (low molecular weight, LMW) (Kreis *et al.*, 1985).

Two to six antibodies from each of four specificity classes were used:

(a) High-mobility Gliadin Binding

221/23, IgG₁ (α - γ -gliadin, C-LMW glutenin (LMW-gtn))

222/5, IgG₁ ($\alpha\beta\gamma$ - ω -gliadin, B-, C-LMW-gtn)

227/22, IgG₁ (β - α -gliadin)

230/9, IgG₁ ($\alpha\beta$ -gliadin, B-, C-LMW-gtn)

403/8, IgG₁ ($\beta\gamma$ -gliadin, C-LMW-gtn)

404/6, IgG₁ ($\beta\gamma$ -gliadin, C-LMW-gtn)

(b) *Gamma- and Omega-gliadin and Glutenin Binding*218/17, IgG₁ ($\gamma\omega$ - > β -gliadin, B-LMW-gtn > HMW-gtn)237/24, IgM ($\gamma\omega$ -gliadin, HMW- and B-LMW-gtn)246/21, IgM ($\gamma\omega$ -gliadin, HMW- and B-LMW-gtn)(c) *Omega-gliadin and Glutenin Binding*304/13, IgG₁ (ω -gliadin, HMW-gtn > B-LMW-gtn)401/21, IgG₁ (ω -gliadin, HMW-, B- and C-LMW-gtn)405/7, IgG₁ (ω - > γ gliadin, HMW-, B- and C-LMW-gtn)(d) *Broad-specificity Antibodies*

243/11, IgM, 243/12, IgM, 246/22, IgM

Cell lines, after 2–3 cloning cycles by limiting dilution, were grown as ascites tumours in BALB/c mice for 10–12 days. Harvested ascitic fluids were treated with saturated ammonium sulphate, pH 7.0, to a final concentration of 45%; the precipitate from centrifugation was redissolved in 10 mM-sodium phosphate, pH 6.8, and dialyzed against the same buffer. Monoclonal antibodies were purified by hydroxylapatite (BioRad, Richmond, CA, USA) adsorption chromatography (Stanker *et al.*, 1985) using elution with one column-volume each of 10 mM-sodium phosphate (pH 6.8), 50 mM-sodium phosphate; then a nine column-volume gradient of sodium phosphate buffer, 50- to 300-mM final concentration, pH 6.8. Antibodies were typically over 90% pure upon analysis by sodium dodecyl sulphate–polyacrylamide gradient gel electrophoresis (Gradipore gels, Pyrmont, Australia). Monoclonal antibodies were labelled with horseradish peroxidase by a modification of the method of Nakane & Kawoi (1974); conjugates were freed of unreacted enzyme by precipitation with 50% ammonium sulphate. Only conjugates which had titres greater than 1/1000 in direct ELISA using 2 μ g per well gliadin were used; therefore, conjugates of 122/24, 139/18 and 246/21 were rejected for this study.

Crude gliadin was prepared by extraction of wheat flour (cv. Timgalen) by treatment with 70% (v/v) ethanol, following 10% (w/v) NaCl extraction, as described earlier (Skerritt & Underwood, 1986).

Sequential Sandwich Enzyme-linked Immunosorbent Assay (ELISA)

Microwell plates, made of either PVC (Dynatech, Chantilly, VA, USA) or polystyrene (Immulon II, Dynatech; or Nunc Maxisorb, Roskilde, Denmark) were used; the former were U-bottomed and the latter flat-bottomed. Microwells were coated with 1 μ g purified monoclonal antibody in 100 μ l/well of 50 mM-sodium carbonate buffer, pH 9.6, for 1 h at 20°C. In some experiments, different coating times or temperatures were used (as indicated). Following three washes with phosphate buffered saline (PBS) (50-mM sodium phosphate buffer, pH 7.2, containing 0.9% NaCl–0.05% Tween 20, the blocking solution (1% bovine serum albumin (BSA) in PBS) was added and incubated for 1 h at 20°C. Following three washes, antigen was added, diluted in 100 μ l 1% (w/v) BSA–PBS–0.05% (v/v) Tween 20. Gliadin antigens were diluted from a 70% (v/v) ethanol stock, and glutenins from a 100 mM-KOH stock; the highest concentrations of either vehicle used (4%) did not inhibit antibody binding. After 30 min incubation at 20°C, unbound antigen was removed by three washes, and HRP-labelled monoclonal antibody was added, diluted in 100 μ l 1% BSA–PBS–0.05% Tween 20. The concentration of labelled antibody was chosen such that it gave a product absorbance of 0.8–1.2 using an indirect ELISA with 2 μ g per well gliadin or glutenin. Following 30 min at 20°C, microwells were washed three times and 100 μ l substrate (0.003% H₂O₂, 3-azino bis-3-ethylbenzthiazoline-6-sulphonic acid in 100 mM-sodium citrate, pH 4.5) added

for 20 min at 20°C. Reactions were terminated by acidification and product absorbance was determined at 414 nm.

In all of the two-site MAb combinations investigated, labelled antibodies were incubated in parallel (after antigen incubation) on a blocked plate lacking capture antibody. At very high antigen or tag antibody concentrations, some colour development was noted. These absorbance values were subtracted from those obtained in the presence of various capture antibodies.

Simultaneous Two-site Enzyme-linked Immunosorbent Assay (ELISA)

The method used was as for the sequential assay, with the exception that 50 μ l enzyme-labelled antibody was added to blocked and antibody-coated polystyrene microwells immediately followed by 50 μ l antigen solution. Plates were shaken gently, then incubated for 30 min at 20°C.

Competition ELISA

Immulon microwell plates were coated (8 h at 37°C) with 2 μ g gliadin per well, in 100 μ l 50 mM-sodium carbonate buffer, pH 9.6, containing 3% ethanol. Following three washes in PBS-0.05% Tween and blocking, wells were washed once before 50 μ l gliadin diluted in 1% BSA in PBS-0.05% Tween was added to appropriate microwells. Aliquots of 50 μ l of antibody, diluted in 1% BSA-PBS-0.05% Tween, were immediately added, and the plate contents gently mixed and incubated for 30 min at 20°C. After four washes, microwells were incubated with 100 μ l HRP-labelled rabbit anti-mouse immunoglobulins (DAKO, Denmark), diluted 1/5000 in 1% BSA-PBS-0.05% Tween for 30 min at 20°C, before four further washes and substrate addition. Where labelled monoclonal antibodies were used, the second antibody incubation was deleted. Monoclonal antibody concentrations were selected to yield an absorbance of 0.8-1.0 in the absence of competing gliadin.

RESULTS

Comparison of PVC and Polystyrene Solid Phases

In the construction of each two-site assay, parallel incubations were performed using microwells lacking coating (capture) antibody. In the absence of coating antibody on polystyrene plates, little enzyme-labelled antibody binding was noted. However, on PVC microwells, the binding of each of several HRP-labelled antibodies (221/23, 222/5, 122/24, 243/11) and alkaline phosphatase-labelled antibodies (221/23, 222/5 and 122/24) was almost equivalent to that seen in the presence of capture antibody. The nature of this non-specific binding to PVC microwells was investigated further using MAb 222/5 and its HRP conjugate (Figure 1(a)). This antibody, with rather broad gliadin specificity, is able to function simultaneously as capture and tag antibody. Gliadin-antigen concentration curves were prepared using both PVC and polystyrene (Immulon) microwell plates, and several blocking agents were investigated: (a) 1% BSA in PBS; (b) 5% BSA in PBS; and (c) 0.5% skimmed milk powder in 0.9% NaCl-10 mM-Tris-HCl, pH 7.6). The latter two agents are more extensive blockers (Kenna *et al.*, 1985). Further, gliadin antigen was diluted in several buffers: (a) 1% BSA in PBS-0.05% Tween 20; (b) 'a' containing 5% BSA; (c) 'a' containing 650 mM-NaCl; (d) PBS-0.05% Tween containing 650 mM-NaCl; (e) 'd' containing 2% Tween; and (f) 0.5% non-fat dried milk in 0.9% saline-10 mM-Tris-HCl, pH 7.6.

Alteration of blocking agent (alone) did not reduce the high non-specific binding observed on PVC plates with antigen diluted in 1% BSA-PBS-0.05% Tween. However, the use of milk-based diluent on 1% BSA-blocked plates (Figure 1(b)) or 5% BSA (on milk-blocked plates), together with high salt and Tween diluent, with either milk

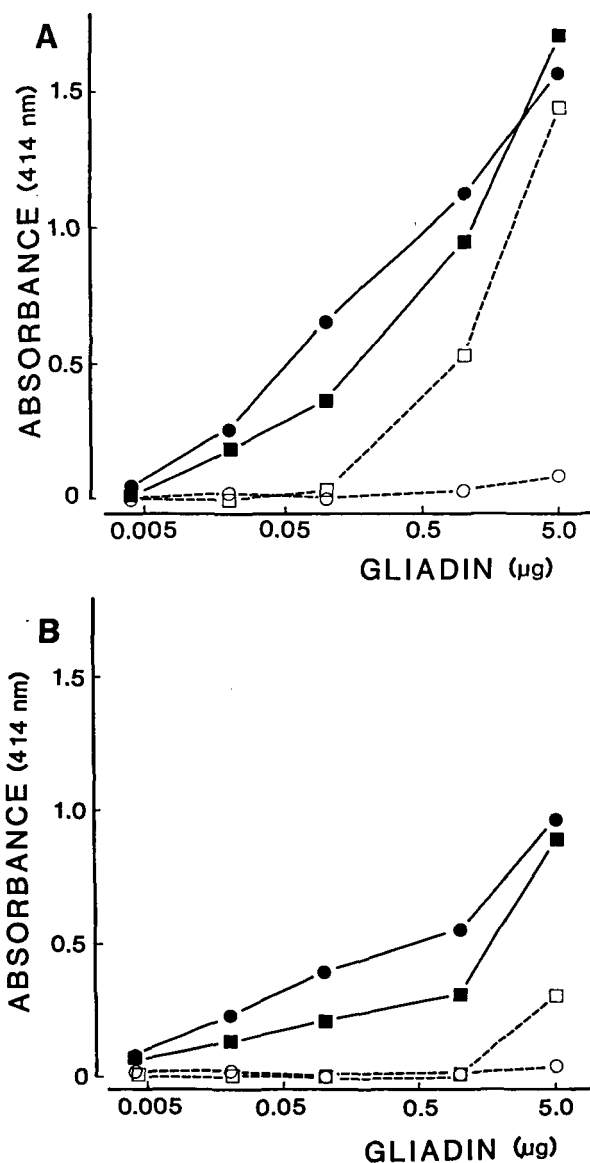


FIG. 1. Effects of antigen and antibody diluent and microwell plate type on antibody performance in sandwich ELISA: (●, ○) polystyrene, (■, □) PVC microwells; closed symbols with capture antibody (222/5), open symbols without capture antibody; (A) antibody 222/5-HRP with standard diluent and blocking conditions (1% BSA-0.05% Tween in PBS); (B) antibody 222/5-HRP with stringent diluent blocking conditions (0.5% milk powder-10 mM-Tris-HCl, pH 7.6, 0.9% NaCl).

or BSA blocker, decreased non-specific binding to PVC plates considerably. Under these more stringent blocking conditions, ELISA product absorbance values obtained with both PVC and polystyrene microwells were suppressed. Accordingly, Immulon polystyrene microwells were used, with a 1% BSA in PBS blocking agent and a 1% BSA-PBS-0.05% Tween diluent, for subsequent experiments. Another brand of poly-

styrene microwells (Nunc) performed equivalently, indicating that these results were not confined to Dynatech-brand microwells.

Effects of Capture Antibody Coating Time, Temperature and Concentration

These effects were investigated using monoclonal antibody 401/21 as both capture and labelled 'tag' antibody. Antigen concentration vs absorbance plots were constructed using 10 $\mu\text{g}/\text{ml}$ capture antibody, coated for either 0.25, 1, 2 or 18 h at either 20°C or 37°C. A combination of 1 h coating time and the lower temperature produced the best results: maximal absorbance values obtained with 18 h coating at 37°C were 40% lower (Figure 2). Increasing the incubation time for antigen and for labelled antibody from 30 to 60 min increased the absorbance response at high antigen concentrations for each coating condition studied. A concentration of 10 $\mu\text{g}/\text{ml}$ capture antibody also appeared to be optimal for most antibodies used in the study. In general, the higher concentration of capture antibody (100 $\mu\text{g}/\text{ml}$) produced a slightly lower colour development, while at 1 $\mu\text{g}/\text{ml}$ significantly less colour was noted. Only very slight colour developed at 100 ng per well (Figures 3(a), (b)). From information on the binding capacity of IgG to polystyrene, it is likely that the solid phase is saturated above 10 μg per well (Cantarero *et al.*, 1980; Butler, 1981). Increases in labelled antibody concentration produced an upward shift in the antigen/absorbance response curve, especially at high antigen concentrations (results for 10 $\mu\text{g}/\text{ml}$ capture antibody are depicted in Figure 3(b)). A low-capture antibody concentrations, increasing labelled antibody had little effect, presumably due to a limitation in the amounts of captured antigen.

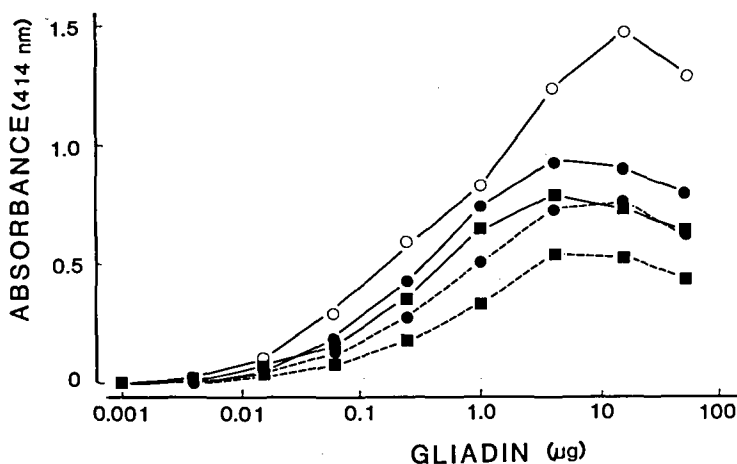


FIG. 2. Effects of assay conditions on gliadin detection using antibody 401/21 as both solid-phase bound and labelled antibody: (●, ○) 20°C coating; (■) 37°C coating. Solid lines represent 60 min coating and broken lines overnight coating. The open symbols depict results from an experiment using 60 min antigen (rather than 30 min) and 60 min labelled antibody incubations.

Capture and Tag Antibody Combinations

Capture antibodies from the α - or β -gliadin-specific group did not function when the same antibody was used as labelled 'tag' antibody (Table 1). In contrast, the γ -gliadin binding members of the group, 403/8, 404/6 and 222/5, functioned with most gliadin-binding tag antibodies tested. However, many high-mobility gliadin binding anti-

bodies were able to function in two-site assays with antigen and the broad-specificity antibody 243/11, and in most cases with the γ - or ω -gliadin and high molecular weight glutenin-binding antibodies 218/17, 237/24, 304/13 and 401/21.

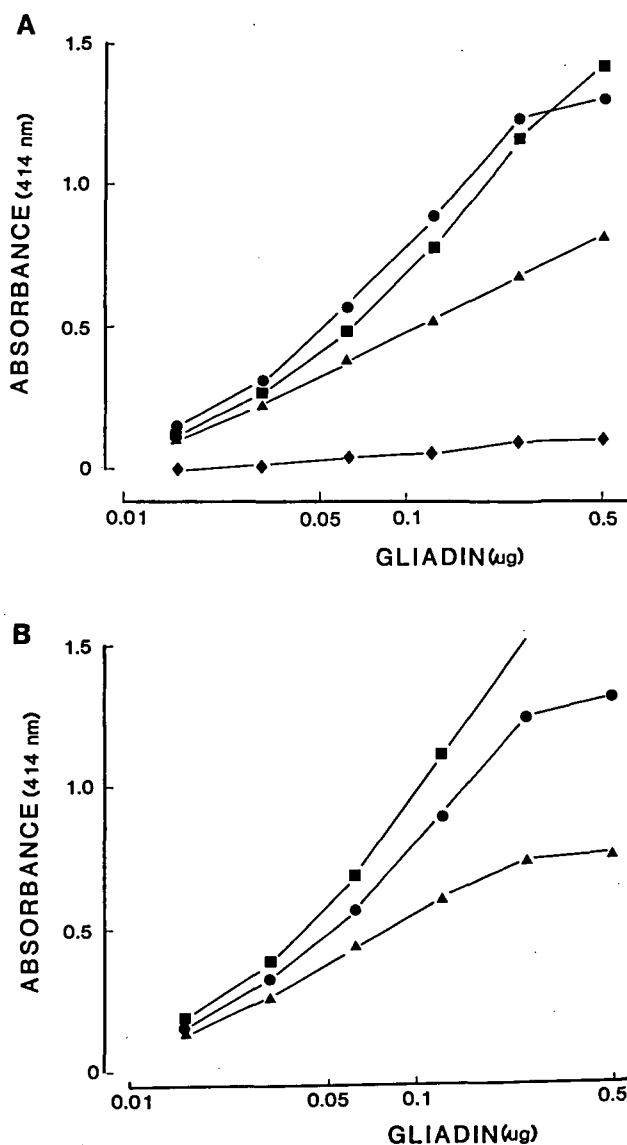


FIG. 3. Effects of solid-phase (A) and labelled (B) antibody concentrations on sandwich assay using 401/21; A: (♦) 0.1, (▲) 1.0, (●) 10, (■) 100 $\mu\text{g}/\text{ml}$ capture antibody; B: (■) 1/3500, (●) 1/7000, (▲) 1/14000 dilution of tag antibody.

Most γ - and ω -gliadin-binding capture antibodies did not form complexes with labelled α - and β -gliadin antibodies such as 221/23 and 230/9, although some of the γ -gliadin-binding antibodies, which cross-react with β -glutens at high concentrations (Skerritt & Underwood, 1986) formed 'sandwich' complexes with the β -gliadin bind-

ing antibody 227/22-HRP. Each antibody was tagged by the broader-specificity antibody 222/5, which weakly binds ω -gliadins and more strongly binds α -, β - and γ -gliadins. The very high affinity ω -gliadin antibody 304/13 formed weak complexes with HRP-labelled α - and β -gliadin binding antibodies, when 1 μ g gliadin was used. Unlike the α - and β -gliadin-binding antibodies, the γ - and ω -gliadin binding antibodies functioned with the same antibody as capture and tag, as well as with broad-specificity tag antibodies. As would be expected, the broad specificity capture antibodies 243/11 and 243/12 functioned with virtually all tag antibodies tested, including themselves. A good tag antibody was 246/22 but it captured very weakly, and with only three antibodies (Table 1).

'Simultaneous' Sandwich ELISA

The effects of sequential incubation of antigen and labelled antibody were compared with simultaneous incubation, using each possible combination of antibodies 222/5, 304/13 and 401/21 as capture or tag. In each case, the shape of the antigen concentration curve was altered by simultaneous incubation. For antibodies 304/13 and 401/21, the curves became bell-shaped rather than sigmoidal, and the maximal absorbance was considerably lower (Figure 4). Colour development was relatively low for combinations involving 222/5 as capture antibody, thus a maximum absorbance was not reached in either assay format. The sensitivity of antigen detection (detectability) was slightly higher for the simultaneous assay when 401/21 was used as the tag antibody, but slightly (or considerably) lower when 222/5 and 304/13 were used as tag antibodies, respectively. Similar results were obtained when a threefold higher concentration of 401/21-HRP was used, except that maximal absorbances were greater and the peak or plateau of the curve was shifted to higher gliadin concentrations.

Comparison of Antigen-competition and Sandwich Assays

Results with increasing concentrations of competing gliadin antigen for eight monoclonal antibodies are shown in Figure 5. All antibodies depicted are of the IgG₁ isotype; several IgM monoclonal antibodies (including 237/24, 246/21, 243/11 and 243/12, see Table 1) showed little or no inhibition up to the limit of gliadin solubility in the buffers used. Inhibition potencies varied considerably; the most potent inhibition was displayed by 222/5 (competition 50% inhibitory gliadin concentration, $IC_{50}=0.2$ μ g/ml) and the least potent by 405/5 ($IC_{50}=100$ μ g/ml). The slopes of the gliadin inhibition curves also differed for different monoclonal antibodies. Some of these antibodies were assessed in the two-site assay (Figure 6), with assays using the same antibody combination as capture and tag antibody. Some antibodies such as 221/23 and 230/9, which showed potent gliadin inhibition in the competition assay, did not function in this manner in the sandwich assay. However, some IgM antibodies such as 237/24 and 243/11 performed well in the sandwich assay format. A comparison of the competition 50% inhibitory gliadin concentration (IC_{50}) and the concentration producing 50% maximal colour development in the sandwich assay (EC_{50}) showed that antibodies could either be less sensitive (e.g. 222/5: $EC_{50}=30$ μ g/ml, $IC_{50}=0.3$ μ g/ml; 404/6: $EC_{50}=10$ μ g/ml, $IC_{50}=3.6$ μ g/ml), equally sensitive (e.g. 403/8: $EC_{50}=12$ μ g/ml, $IC_{50}=12$ μ g/ml) or more sensitive (e.g. 401/21: $EC_{50}=4$ μ g/ml, $IC_{50}=12$ μ g/ml) for gliadin detection in the two-site assay.

DISCUSSION

Polystyrene and PVC microwell plates are often used interchangeably in solid-phase immunoassay. The former have the advantages of low price and mechanical rigidity, while several groups have found the latter to bind protein antigens more avidly

TABLE 1. Microwell-bound (capture) and enzyme-labelled (tag) antibody combinations: reaction with gliadin

Capture antibody	Enzyme-labelled (tag) antibody													
	High-mobility gliadin binding						Low-mobility gliadin binding					Broad-specificity		
	221/23	222/5	227/22	230/9	403/8	404/6	218/17	237/24	304/13	401/21	405/7	243/11	243/12	246/22
$\alpha\beta$-Gliadin binding														
221/23	—	+	+	+++	++	++	—	+++	+	+++	+++	+++	+++	—
222/5	+++	++	++	+++	+++	++	++	++	+	+++	+++	+++	+++	n.t.
227/22	—	++	—	++	++	++	++	++	+	+++	+++	++	+++	n.t.
230/9	+++	+++	++	—	+++	+++	+++	++	+	+++	+++	+++	+++	n.t.
403/8	—	+++	+	—	++	++	+++	+++	+	+++	+++	+++	+++	—
404/6	+	+++	+	—	++	+++	+++	+++	+	+++	+++	+++	+++	—
$\gamma\omega$-Gliadin/glutenin binding														
218/17	—	+++	++	—	++	++	+	++	n.t.	++	+++	+	+++	+++
237/24	—	+++	+	—	+++	+++	+++	+++	++	+++	++	++	+	n.t.
246/21	—	+++	+	—	++	++	n.t.	—	n.t.	+++	+++	+++	+++	+++
ω-Gliadin/glutenin binding														
122/24	—	+	—	—	n.t.	n.t.	+	n.t.	n.t.	++	n.t.	+++	+++	+++
139/18	—	+	—	—	++	++	++	+++	+++	+++	+++	n.t.	++	—
304/13	++	++	+	n.t.	+++	+++	+++	+++	+++	+++	+++	++	+	—
401/21	—	++	—	—	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
405/7	—	+++	—	n.t.	+	++	+++	++	+	+++	+++	—	++	—
Broad-specificity														
243/11	++	+++	+	+	+++	+++	+++	++	++	++	+++	+++	+++	+++
243/12	+	+++	+	—	+++	++	+++	+	+	++	+++	+++	+++	+++
246/22	—	—	—	—	—	+	+	—	—	—	++	—	—	—

ELISA product absorbance obtained with 1 μ g gliadin: (—) <0.1; (+) 0.1–0.3; (++) 0.3–0.5; (+++) >0.5; (n.t.) not tested.

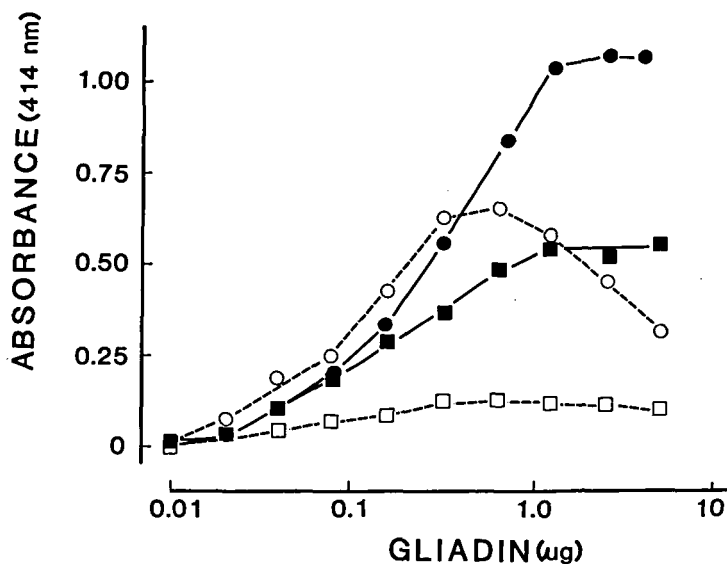


FIG. 4. Comparison of sequential (closed symbols and solid lines) and simultaneous (open symbols, broken lines) sandwich assays using: (●, ○) 401/21; (■, □) 304/13.

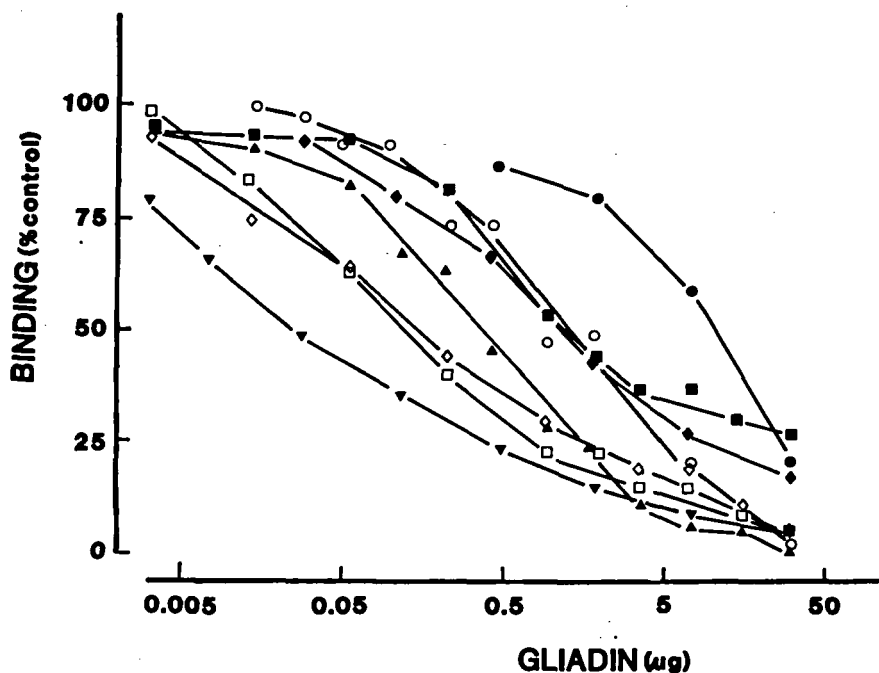


FIG. 5. Antigen-competition ELISA using various monoclonal antibodies: (◇) 218/17; (□) 221/23; (▼) 222/5; (■) 230/9; (◆) 401/21; (○) 403/8; (▲) 404/6; (●) 405/7. Data shown are inhibition of antibody binding by various gliadin concentrations (%).

(Zollinger *et al.*, 1976; Frankel & Gerhard, 1979). This may be at the risk of well-to-well variation in binding and 'edge effects'. Caution must also be exercised when using PVC microwells in sandwich assays with hydrophobic antigens such as gliadins, as considerable non-specific binding of antigen to blocked microwells can occur. Stringent blocking conditions, which reduced this non-specific binding, also markedly reduced the ELISA signal.

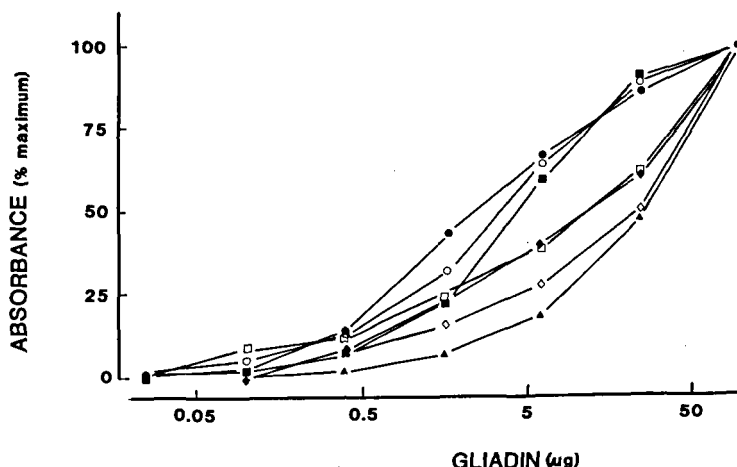


FIG. 6. Sandwich ELISAs using the same antibody pair as solid-phase bound and labelled partner; (▲) 222/5; (●) 237/24; (◇) 243/11; (○) 304/13; (■) 401/21; (□) 403/8; (◆) 404/6.

Brief descriptions of sandwich enzyme immunoassays for gliadin have appeared in the literature (Lane *et al.*, 1982; Windemann *et al.*, 1982; McKillop *et al.*, 1985; Troncone *et al.*, 1986; Freedman *et al.*, 1987; Ayob *et al.*, 1988; Mills *et al.*, 1989). However, all of these workers have used a polyclonal antibody as the capture and/or tag antibody. The studies described in this paper have used monoclonal antibody combinations instead, and the incubation conditions have been optimized. A rather large proportion of the monoclonal antibodies tested functioned in the two-site assay format. Of the 17 capture antibodies tested, 16 functioned well; the antibody that functioned very poorly was a broad-specificity IgM. Presumably this antibody adopted an inactive conformation on polystyrene surfaces (Dierks *et al.*, 1986), as enzyme conjugates of the same antibody were useful 'tag' antibodies. Other antibodies used were of the IgG₁ isotype, except for 237/24, 246/21, 139/18, 243/11 and 243/12 (all IgM); with the exception of 246/22, both of these isotypes functioned reasonably well as capture or tag antibodies.

For a single monoclonal antibody to be able to function as both capture and tag antibody in a two-site ELISA, epitopes recognized by the antibodies must be present in multiple copies in the antigen. As each of the antibodies used in this study bound to SDS-denatured proteins (Skerritt & Underwood, 1986; Donovan *et al.*, 1989), it is likely that such epitopes in gluten proteins are typically linear. Therefore, the results are in keeping with the known presence of repeating sequences in many families of gluten proteins. Each of the γ - and ω -gliadin-binding, glutenin-binding and broad-specificity capture antibodies (except 246/22) functioned when the same antibody was used as an enzyme tag. The groups of proteins (the γ - and ω -gliadins) are known to contain a greater proportion of internal repeat sequences than the α/β -type gliadins

(Kreis *et al.*, 1985). In contrast, several α/β gliadin-binding antibodies with narrower specificity did not function simultaneously as capture and tag antibodies in two-site assays, suggesting that they bound to unique epitopes.

Another reason why a particular pair of two different monoclonal antibodies may fail to function is that they may recognise different components in a complex mixture of proteins such as gliadin. For example, most γ - and ω -gliadin-specific capture antibodies were not 'tagged' by α/β -gliadin specific antibodies such as 221/23 or 230/9. The ability of γ/ω -gliadin binding antibodies such as 218/17, 237/24, 304/13 and 401/21 to produce significant colour with 1 μ g gliadin in combination with some of the α or β -gliadin-binding capture or tag antibodies may be due either to the higher affinities of some of these tag antibodies or possibly be a result of binding to LMW glutenins, found in small quantities in 'gliadin' fractions (Bietz & Wall, 1973). More likely, it is a function of the very high affinities of this group of capture antibodies. The specificities of monoclonal antibodies to homologous protein families in dependent on antibody concentration (Mossman *et al.*, 1980; Nigg *et al.*, 1982). For example, MAb such as 221/23 or 230/9 bind α - and β -gliadins at nanogram antigen concentrations or lower, but at microgram concentrations they will bind γ - and ω -gliadins and glutenins. Thus, at the microgram level of antigen used for Figure 1, cross-reactions with tag antibodies binding the latter protein fractions were apparent, but not at lower concentrations (not shown). At 100-fold lower gliadin concentrations these combinations produced little colour, while combinations of $\alpha/\beta/\gamma$ -gliadin-binding capture and tag or γ/ω -gliadin-binding capture and tag still functioned well.

It proved possible to develop assays incorporating simultaneous incubation of antigen and labelled antibody. However, both the lower maximal absorbances plus the biphasic antigen response curves obtained with simultaneous assays would suggest that sequential assays, yielding sigmoidal curves, are preferable for gliadin quantification. The concentration vs response curves for both the competition and two-site assays were not parallel for the different antibodies. While the relative affinities of the different antibodies for free antigen may relate to the competition. IC_{50} values (Nieto *et al.*, 1984; Rath *et al.*, 1988) recruitment of weak cross-reactions at high antigen or antibody concentrations (Skerritt & Underwood, 1986) and presence of multiple copies of epitopes would also contribute to differing slopes. Although others have attempted to do so with polyclonal antisera to gliadin (Troncone *et al.*, 1986), it did not prove possible to make a general conclusion on the relative sensitivities of two-site and competition ELISAs, as some particular antibodies functioned far better in one format than the other. Nevertheless, some capture and tag antibody combinations, e.g. 221/23 and 222/5, were highly sensitive, detecting gliadin below 500 μ g/well.

The sandwich assay has the advantage that sensitivity can often be improved by increasing labelled MAb concentrations (Clark & Engvall, 1980; Tijssen, 1985), and, because excess capture and tag are usually used, results are less dependent on small changes in coating antigen or antibody concentrations (Tijssen, 1985). Results are also not affected by possible changes in conformation of the antigen upon adsorption to the solid phase (Friguet *et al.*, 1984). However, because two-site assays rely on the interaction of pairs of antibodies, their specificity characteristics can be less readily predicted from indirect ELISA or blotting data. The specificities of monoclonal antibody based two-site assays for gluten proteins are examined in the accompanying paper (Skerritt *et al.*, 1989).

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