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Cytomegalovirus Glycoprotein B-Specific Antibody Analysis Using Electrochemiluminescence Detection-Based Techniques

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An electrochemiluminescence technique was used to develop versatile and sensitive assay strategies for determination of seroreactivities against biologically important cytomegalovirus neutralization epitopes expressed on glycoprotein B. Indirect binding assays showed wide linear assay ranges and revealed that serum samples diluted in parallel with a monoclonal antibody-based standard, simplifying quantitative analytical assessments.

Cytomegalovirus (CMV) is a commonly occurring virus which under certain circumstances (in immunocompromised and neonatally infected individuals) may manifest itself with serious disease symptoms. Healthy infected individuals, though, normally do not experience any sequelae as a consequence of persistent infection since their immune systems restrict the viral infection. Both cellular and humoral immune mechanisms appear to be involved in this activity, and the potential for use of either vaccination or passive immunization regimes to combat serious CMV-related diseases has been evaluated (10, 18, 19). A major part of the virus-neutralizing antibody response is directed toward glycoprotein B (gB) (gpUL55) (3). This antigen, which consists of two polypeptide chains that were derived from a common precursor, expresses conserved neutralization determinants in both its N-terminal (epitope AD-2) and C-terminal (epitope AD-1) fragments. Lately, both the fine specificity and the neutralizing capacity of various murine and human antibodies have been delineated (for a review, see reference 24). It has been established that these neutralization epitopes are actually composed of a series of overlapping subepitopes (21, 23) and, at least in the case of AD-1, that only a fraction of the high-affinity antibodies against that epitope will actually mediate virus neutralization (21). Since gB is a molecule that has been investigated in various vaccine trials (1, 6, 17), it would be of value to efficiently monitor the developing humoral immunity repertoires against its epitopes. Consequently, we have developed a versatile and sensitive assay based on the recently developed electrochemiluminescence (ECL) approach (27) to characterize antibody responses against these epitopes. This technology relies on detection of ruthenium(II)-Tris-bipyridine chelate (TAG)-labelled molecules (e.g., antibodies) that bind to magnetic beads through, e.g., a specific immunological interaction. The approach offers such advantages as extensive linear assay ranges, good signal-to-noise ratios, and high sensitivity.

When assessing the potential of the ECL technique by using the ORIGEN analyzer (IGEN Inc., Rockville, Md.) to detect antibodies against CMV gB AD-1 and AD-2, the following conditions were employed. We selected for use as the antigen in the assay for AD-1 reactivity the recombinant fusion protein NRD1-BCCP (22), which is composed of residues 541 to 640 of CMV (strain AD169) gB and a fragment of the biotin carboxyl carrier protein (BCCP) subunit of acetyl coenzyme A carboxylase (9). The latter protein moiety is biotinylated by the bacterial host, thus enabling the product to bind to streptavidin-coated magnetic beads. The AD-2-expressing antigen was synthesized as a biotinylated 20-mer peptide (T7-13B) (16) by standard solid-phase techniques (20). Magnetic particles (2.8-μm-diameter Dynabeads) coated with streptavidin were obtained from Dynal A/S (Oslo, Norway). Antibodies were labelled with *N*-hydroxysuccinimide ester-activated TAG (IGEN Inc.) according to the manufacturer's recommendations. Five to eight moles of the fluorochrome was incorporated per mole of antibody. In the basic analysis of monoclonal antibody reactivity, streptavidin-coated magnetic beads (25 μl; 0.5 to 1 mg of beads/ml) were incubated with biotinylated antigen (e.g., 25 μl of T7-13B at 250 ng/ml), specific antibodies (25 μl), and TAG-labelled sheep anti-human immunoglobulin G (IgG) (25 μl at 1 μg of IgG/ml) for 30 min at room temperature with constant rotation. In some experiments, streptavidin-coated beads had been precoated with biotinylated antigen and excess antigen was removed by washing prior to addition of antibody samples. All components were diluted in assay diluent containing gelatin (IGEN Inc.). Following addition of assay buffer (IGEN Inc.), the fluorescence bound to the magnetic beads was determined with the ORIGEN analyzer (IGEN Inc.) as described previously (13). Samples were analyzed in duplicate. The coefficient of variation of duplicate samples was typically below 10%. Background signals in the absence of monoclonal antibody were subtracted before further calculations were performed.

In order to assess the ability of the ECL technique to detect antibodies against CMV gB AD-1 and AD-2, we initially evaluated its performance with a number of specific human monoclonal antibodies (16) against these epitopes. High-affinity human antibodies specific for various substructures of the AD-1 epitope (21) recognized the recombinant fusion protein NRD1-BCCP bound to streptavidin-coated magnetic beads (Fig. 1). The slopes of their reactivities were linear (but slightly below 1) and essentially parallel to each other. However, the maximum signal achievable with the different antibodies varied somewhat; for instance, ITC52 did not reach the same maximum signal level as ITC48. Similarly, a human antibody specific for AD-2 bound to peptide T7-13B (Fig. 2) but not to irrelevant biotinylated peptides. Irrelevant monoclonal antibodies specific for CMV pp65 (15), for instance, did not bind

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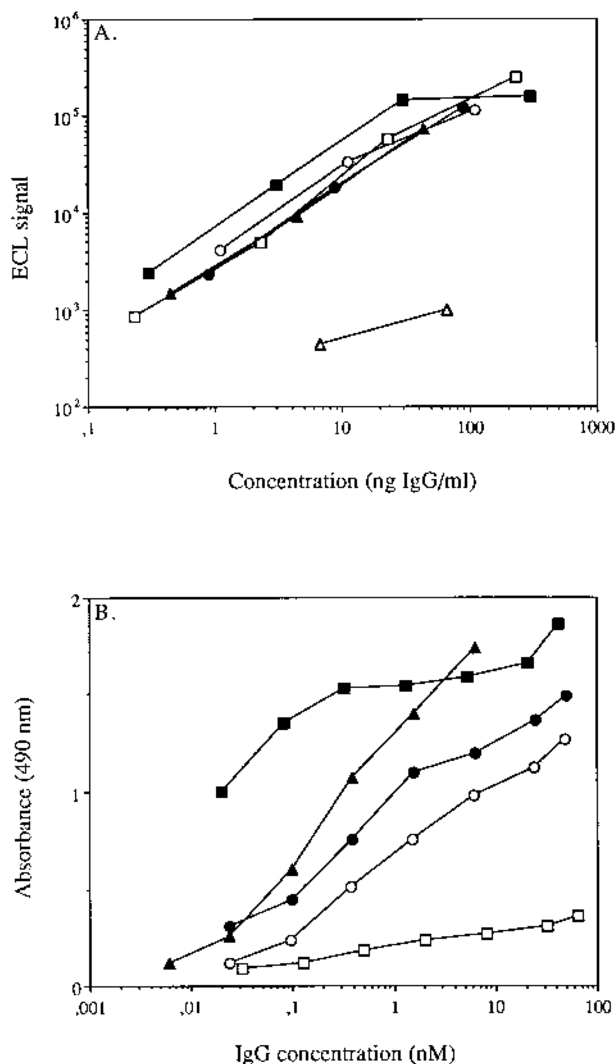

FIG. 1. (A) Reactivity of human gB (AD-1)-specific antibodies ITC33 (○), ITC39 (●), ITC48 (□), ITC52 (■), and ITC63B (▲) to recombinant antigen NRD1-BCCP bound to streptavidin-coated magnetic beads as determined by ECL. Human antibody ITC88 (△) specific for AD-2 served as a negative control. Four antibodies against CMV pp65 (15) were also negative in this assay (data not shown). Concentrations refer to the original sample, of which 25 μl was used in each assay tube. (B) Reactivity of the same human anti-gB (AD-1)-specific monoclonal antibodies to virus-derived natural gB as determined by ELISA. Antibodies represented by filled symbols bind epitope subtype A, while ITC33 and ITC48 bind subtypes B and E (as defined by Schoppel et al. [21]), respectively. The observed signal reactivity differences are not related to affinity constant differences since ITC48 displays a greater affinity for gB than does either ITC33 or ITC39 (16).

the antigen-coated magnetic beads in either assay. The useful linear range spanned >2.5 orders of magnitude of IgG concentration (Fig. 2) with a sensitivity for AD-2-specific antibodies of less than 2 pg of specific IgG/assay tube. The slope of the dilution curve in the latter case was very close to 1 ($x = 0.987$; standard error of the mean $= 0.023$ [$n = 5$]), and the reactivity of the antibody could be efficiently inhibited by the addition of soluble decapeptides covering the previously reported (14, 16) epitope (Fig. 3). A competition assay based on the principle that unlabelled sample antibody could block the binding of directly TAG-labelled AD-2-specific antibody ITC88 was less sensitive that the direct binding assay using TAG-labelled sheep anti-human IgG as the detection reagent (data not

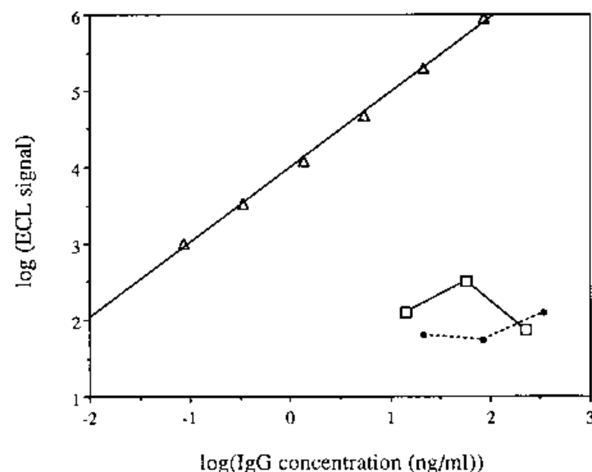

FIG. 2. Reactivity of human gB (AD-2)-specific antibody ITC88 (△) with T7-13B bound to streptavidin-coated magnetic beads as determined by ECL. Human antibodies ITC48 (□) and MO61 (anti-CMV pp65) (dashed line) served as negative controls. Similarly, ITC88 was unable to bind a biotinylated human immunodeficiency virus type 1 V3 epitope-related peptide studied under the same conditions (data not shown). Antibody concentrations refer to the original sample, of which 25 μl was used in each assay.

shown). However, this assay was sufficiently sensitive for determination of biologically significant levels (above the equivalent of 0.6 μg of ITC88/ml of undiluted serum) of AD-2-specific antibodies in serum. The useful assayable concentration range in this assay setup (only one order of magnitude) was not as wide, however, as in the indirect binding assay (Fig. 2).

Serum samples obtained from seropositive and seronegative individuals were assessed for the presence of antibodies against CMV gB epitopes AD-1 and AD-2 by the indirect binding assay (Table 1). As a consequence of the presence of a high level of unrelated antibodies in these samples, unbound antibodies had to be removed by washing (three times with 0.5 ml of phosphate-buffered saline containing 0.1% bovine serum albumin) the beads on a magnetic stand prior to addition of the TAG-labelled sheep anti-IgG preparation. Under such assay conditions it was generally observed that antibodies against epitope AD-1 could be found among all CMV-seropositive individuals in this study (although the level varied 25-fold), in agreement with the observed potent immunogenicity of AD-1 among CMV-infected individuals (7). In contrast, only some of the CMV-seropositive individuals had measurable antibodies against the AD-2 epitope, despite the high sensitivity of the assay. The reactivity of AD-2-specific antibodies in serum could be inhibited by soluble peptides covering the relevant previously defined sequences (Fig. 3), proving the specificity of the assay. Both AD-1- and AD-2-specific antibodies in serum diluted in parallel with standard monoclonal antibodies, thus simplifying relative quantification (Fig. 4). Concentrations of AD-2-specific antibodies determined by competition and indirect binding assays correlated well with each other ($r^2 = 0.94$ for a linear relationship when plotting $\log_{10}$ concentrations of AD-2-specific reactivity as determined by the two techniques) (data not shown). Low levels of AD-1 reactivity could also be found in samples otherwise considered to be CMV seronegative. This may result from cross-reactivity with antibodies specific for gB of other herpesviruses, as significant homology exists at the protein level in the epitope expressed by our recombinant protein and regions considered to be immunoreactive in gB proteins of other herpesviruses (2, 4, 5). Such

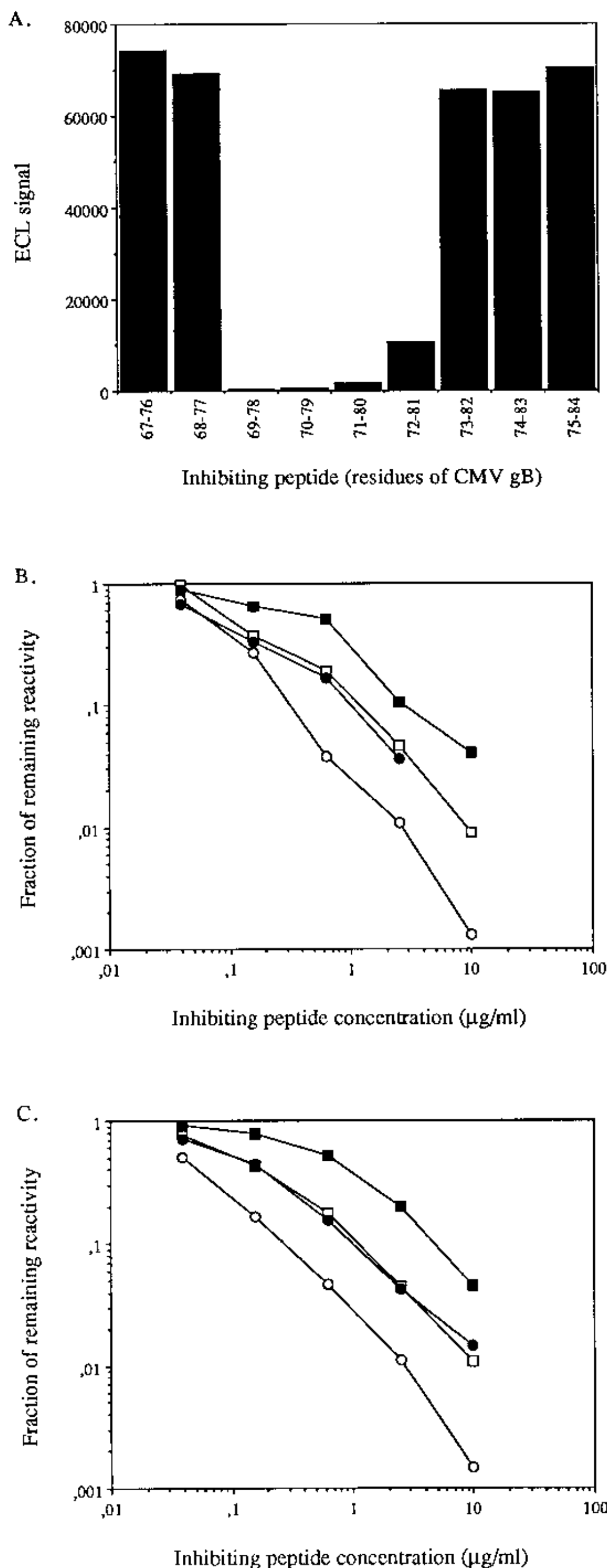

FIG. 3. (A) Inhibition of reactivity of ITC88 with decapeptides (final peptide concentration, 2.5 µg/ml) covering parts of the AD-2 epitope of CMV gB. (B and C) Titration of inhibition of reactivity of ITC88 (open symbols) and two human serum samples (closed symbols) with biotinylated peptide T7-13B (gB epitope AD-2) as determined by ECL technology. Inhibiting synthetic peptides spanned residues 69 to 78 (○, ●) and 70 to 79 (□, ■) of gB. Serum specimens 560 (B) and 594 (C) were used in this study.

TABLE 1. Comparison of serum antibody titers against CMV gB epitopes AD-1 and AD-2 determined by ELISA and ECL technology

Serum specimen no.	CMV result[a]	ELISA absorbance		Antibody titer determined by ECL	
		AD-1[b]	AD-2[c]	AD-1[d]	AD-2[e]
539	+	1.00	>1.6	21	81
557	+	>1.9	>1.9	280	230
560	+	0.75	>1.3	160	240
594	+	>2	>2	720	1,170
619	+	1.11	0.22	110	20
636	+	0.67	<0.10	37	<9
658	+	0.71	<0.10	130	<9
660	+	>2	1.74	510	170
578	−	NA[f]	NA	<9	<9
581	−	1.03	<0.10	32	<9
NN	−	0.41	<0.10	41	<9

[a] As determined by Sundqvist and Wahren (25).
[b] ELISA absorbance values determined by Silvestri et al. (22).
[c] ELISA absorbance values determined by Silvestri et al. (23).
[d] Relative titer against NRD1-BCCP in relation to an ITC48 standard (100 = 1 µg of ITC48/ml of undiluted sample), as determined by the indirect binding assay.
[e] Relative titer against T7-13B in relation to an ITC88 standard (100 = 1 µg of ITC88/ml of undiluted sample), as determined by the indirect binding assay.
[f] NA, not assayable due to the presence of high titers of avidin-specific antibodies, which interfere with this assay.

reactivities may go undetected when natural viral preparations are used in serological assays since these preparations rely heavily on antibodies specific for CMV pp65 and pp150 for detection of positive samples.

The results described above demonstrate the applicability of ECL technology for determination of CMV gB-specific antibodies in biologically relevant amounts. One of the major characteristics of these assays is their sensitivity. The assay for AD-2-specific antibodies, for instance, is approximately 10 times more sensitive than a previously used enzyme-linked immunosorbent assay (ELISA) (16) which is based on the detection of recombinant protein immobilized to microtiter plates (data not shown). Furthermore, the useful linear range in ECL-based analysis is very wide, thus alleviating the necessity of assaying several dilutions of every unknown sample in order to obtain quantitative results. At high antibody concentrations, a difference in linearity between different antibodies contained within the AD-1 structure was observed (Fig. 1). This may be related to an inability of a fraction of the recombinant protein to display all AD-1-related subepitopes as a consequence of improper protein folding occurring in the prokaryotic host. Extensive differences in the ability of high-affinity human antibodies to recognize native CMV gB (Fig. 1) or another *Escherichia coli*-derived recombinant protein expressing AD-1 (data not shown) have similarly been observed with ELISAs. The observed reactivity difference is thus not a characteristic of the ECL technique but is inherent in this particular conformation-dependent antigen system. In comparison to ELISA, the ECL-based assay displayed only small differences in reactivities between various antibody clones (Fig. 1). In dilution experiments based on the ECL technology described here, serum samples diluted in parallel with the standard monoclonal antibody (ITC48) used in these experiments. The absolute levels of antibodies in sera reactive with AD-1 and AD-2 epitopes have not been determined. Rather, we relied on a relative comparison with standard preparations containing a monoclonal antibody. Since many AD-1-specific

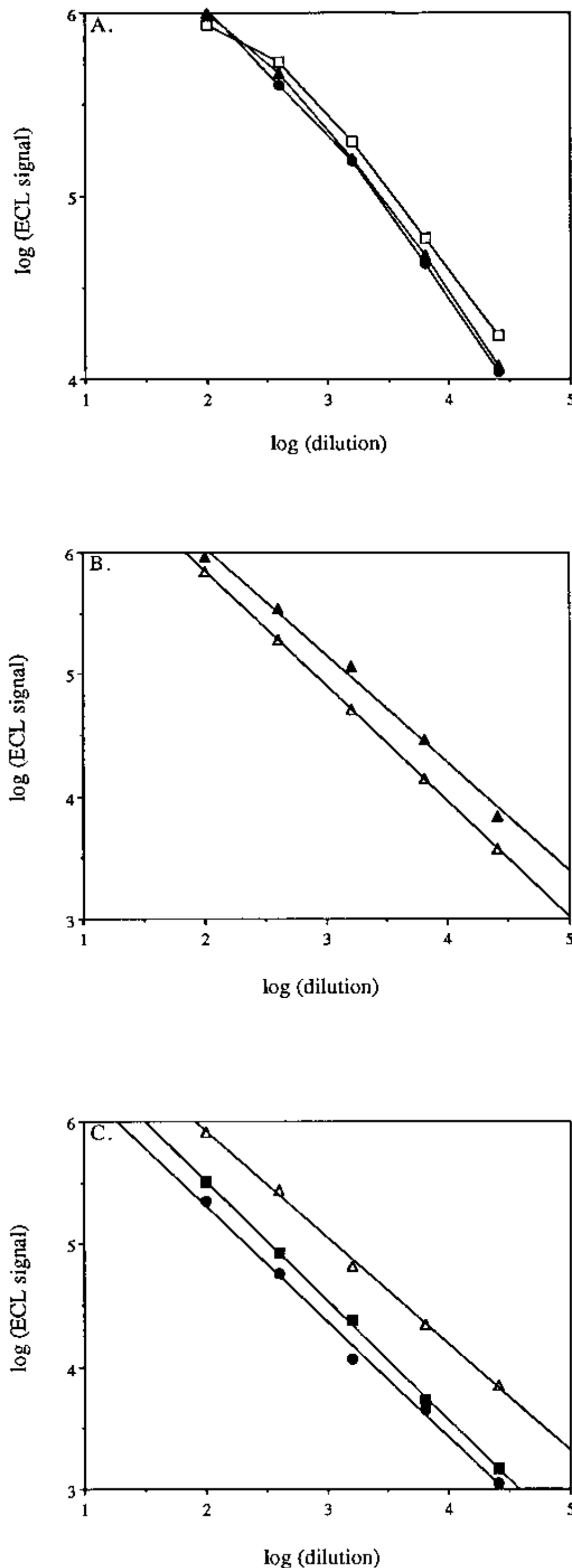

FIG. 4. (A) Reactivity of ITC48 (□) and two human serum specimens (numbers 594 [▲] and 660 [●] [Table 1]) to CMV gB epitope AD-1 as determined in two different experiments using ECL technology. (B and C) Reactivity of ITC88 (△) and three human serum specimens (numbers 560 [■], 594 [▲], and 660 [●] [Table 1]) to CMV gB epitope AD-2 (as expressed by peptide T7-13B) as determined in different experiments using ECL technology.

antibodies are not neutralizing and the neutralizing capacities of others vary considerably (16, 21, 26), in theory no absolute correlation between AD-1-specific seroreactivity in sera and biological activity exists. However, it has been shown in practice that the gB-related specificity best correlating with overall CMV-neutralizing potential in polyclonal antibody preparations was the titer measured against AD-1 (8). Further evaluation of serum samples by the ECL technique will also clarify the relationship between the observed AD-1 titer and the neutralizing capacity with respect to this assay setup.

The fine specificities of antibody repertoires specific for the AD-2 epitope that develop *in vivo* have only recently been investigated (12, 16, 23). Only two human monoclonal antibodies against this epitope have been described in any great detail, and they display very similar specificities in conventional analyses (12, 16). However, serum analysis using immobilized peptides as the antigen (23) suggests that some polyclonal antibody preparations contain AD-2-specific antibodies with slightly different reactivity patterns. Such screening tests can easily be performed by this ECL-based technique, and since the signal correlates in a linear fashion with the relative amount of bound antibody, the ultrafine specificity that develops following infection or vaccination can be efficiently determined. Furthermore, in this study we confirmed that AD-2 specificities could also be assessed in peptide inhibition assays using the ECL technique. In agreement with previous studies (8, 11) reporting a comparatively low immunogenicity for AD-2, we observed that only some individuals actually produced detectable levels of AD-2-specific antibodies. Our data do not specifically confirm the actual ratio of AD-2 responders among CMV-seropositive individuals since some samples were selected on the basis of negative results in AD-2-specific ELISAs. However, these results show that not even a highly sensitive assay is able to detect AD-2-specific antibodies in otherwise CMV-seropositive samples, confirming the poor immunogenicity of this epitope.

Although very little is known about the relationship of ultrafine specificity and/or binding rate constants for the AD-2 epitope to biological activity, those antibodies that have been characterized indeed show similar neutralizing capacities. Serum titers of ITC88-like reactivities close to 100, as defined in this study, would correspond to a potentially functional neutralizing capacity (50% neutralization is observed in vitro at an ITC88 concentration of approximately 1 μg/ml). It will be of interest to evaluate whether gB-based vaccines, which are currently being developed, can be designed to improve AD-2 immunogenicity in order to obtain potentially protective levels in the vaccinated population.

In conclusion, we have shown that ECL-based antibody analysis is a very useful and versatile tool for the evaluation of antibody reactivities specific for biologically important CMV neutralization epitopes. Similar approaches could be taken to evaluate antibody specificities against a variety of other antigens. These assay techniques could be set up either as direct or indirect binding assays, as competitive binding assays, or as antigen inhibition assays to evaluate various specificities of monoclonal and, more importantly, polyclonal antibody samples.

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