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Immunological screening method to detect specific translation products

(solid-phase radioimmunodetection/bacterial colonies/phage λ/autoradiography/β-galactosidase)

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ABSTRACT **We describe a very sensitive method to detect as antigens the presence of specific proteins within phage plaques or bacterial colonies. We coat plastic sheets with antibody molecules, expose the sheet to lysed bacteria so that a released antigen can bind, and then label the immobilized antigen with radioiodinated antibodies. Thus, the antigen is sandwiched between the antibodies attached to the plastic sheet and those carrying the radioactive label. Autoradiography then shows the positions of antigen-containing colonies or phage plaques. A few molecules of antigen released from each bacterial cell generate an adequate signal.**

How can we detect small amounts of proteins made in a bacterial cell without an enzymatic assay? Immunological methods offer an approach: one should be able to detect not only a complete protein, but even a fragment of a protein sequence by virtue of its antigenic determinants. This problem is posed in recombinant DNA experiments in which one might desire to identify a bacterial cell containing a fragment of a gene from a higher cell. If that gene fragment were inserted within a bacterial protein, in phase, antigenic determinants on the higher cell protein could be synthesized and detected. Two immunological screening techniques have been reported (1, 2): they depended on precipitation of the antigen by antibodies included in the agar of the plate or in an agarose overlay. We have devised an extremely sensitive method for screening plaques or colonies that detects antigen-containing areas by a solid-phase "sandwich" assay.

Antibody molecules adsorb strongly to plastics such as polystyrene or polyvinyl and are not significantly dislodged by washing. This is the basis of very sensitive and simple two-site radioimmune assays (3, 4). Thus, we coat a flat disk of flexible polyvinyl with the IgG fraction from an immune serum and press this disk onto an agar plate so that antigen released from bacterial cells during the formation of a phage plaque or through *in situ* lysis of a colony can bind to the fixed antibody. We then incubate the plastic disk with the same total IgG fraction labeled with radioactive iodine so that other determinants on the bound antigen can in turn bind the iodinated antibody. The radioactive areas on the disk expose x-ray film during autoradiography. Since the polyvinyl disk can be thoroughly washed and treated with carrier serum, the background labeling is low; we have detected as little as 5 pg of antigen distributed over an area somewhat larger than a bacterial colony. Microgram amounts of antibody saturate the plastic disk, so an Ig fraction prepared from 5 ml of immune serum will serve to screen 1000 plates of colonies or plaques.

We describe the application of this technique to the detection of an independently assayable protein whose level of production is well characterized and subject to manipulation, *Escherichia coli* β-galactosidase (β-galactoside galactohydrolase; EC 3.2.1.23).

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MATERIALS AND METHODS

Solid-phase radioimmunodetection

Preparation of Solid-Phase IgG. Press 8.25-cm diameter disks cut from clear, flexible polyvinyl, 8 mil thickness (Dora May Co., New York), between sheets of smooth paper to flatten them. Place 10 ml of 0.2 M $NaHCO_3$ (pH 9.2) containing 60 μg of IgG per ml in a glass petri dish and set a flat polyvinyl disk upon the liquid surface. After 2 min at room temperature, remove the disk and wash it twice with 10 ml of cold wash buffer: phosphate-buffered saline (PBS), 0.5% normal rabbit serum (vol/vol), and 0.1% bovine serum albumin (wt/vol). Wash by gently swirling the buffer over the disk, then pouring and aspirating off the wash solution. We coat 10 polyvinyl disks successively with the same IgG solution and use each disk immediately after it is coated.

Release of Antigen from Cells in Bacterial Colonies. Heat induction of λcI857 prophages conveniently lyses cells in colonies (2). After growth of colonies of lysogens for 24 hr at 32°, incubate the plates for 2 hr at 42°.

Alternatively, apply 2 μl of 10 mM $MgSO_4$ containing 10^5 λvir to each colony and leave the plates for 3 hr at 37°, to effect a direct phage-mediated lysis.

Finally, chloroform vapor will lyse the bacteria *in situ*. Place a small volume of chloroform in the bottom of a tightly coverable container and set open petri dishes on a glass support above the liquid. After 10 min, transfer the plates to a desiccator and apply a vacuum to remove any residual chloroform.

Immunoadsorption of Antigen onto Solid Phase. Gently place the IgG-coated surface of a polyvinyl disk in contact with the agar and lysed colonies or the top agar and phage plaques within a petri dish. Smooth any air bubbles that form between disk and agar to the side, since the plastic is flexible. Leave the plates for 3 hr at 4°; then remove the disks and wash them three times with 10 ml of cold wash buffer, aspirating off any adhering cellular material during the washing.

Reaction of ^{125}I-Labeled Antibodies with Solid-Phase Antigen and Autoradiography. Pipet 1.5 ml of wash buffer containing 5×10^6 cpm (γ emission) of ^{125}I-labeled IgG (^{125}I-IgG) onto the center of an 8.25-cm diameter flat disk of nylon mesh (carried by most fabric stores) placed in the bottom of a petri dish. The mesh serves a necessary spacer function. Set a polyvinyl disk on the mesh and the solution so that the entire lower polyvinyl surface is accessible to the radioactive antibody. By repeating the layering process, generating a stack of alternating nylon mesh and polyvinyl disks, 15–20 polyvinyl disks

Abbreviations: PBS, phosphate-buffered saline; wash buffer, PBS containing 0.5% normal rabbit serum and 0.1% bovine serum albumin; ^{125}I-IgG, ^{125}I-labeled IgG.

can be incubated in a single petri dish with 5×10^6 cpm of ^{125}I-IgG each. Incubate overnight at 4°; then wash each disk twice with 10 ml of cold wash buffer and twice with water. Lightly blot the disks to remove water droplets and let them dry at room temperature. Finally, autoradiograph the disks with either Kodak No Screen film or Kodak X-OMAT R film and a Du Pont Cronex Lighting Plus intensifying screen (5).

Procedures specific for β-galactosidase detection

Plating of Bacteria and Phage. Colonies of FMA-10 (W3102 *r*$^-$ *thy*$^-$; from F. Ausubel) (λcI857) or RV(Δ*lac*) (λcI857) were grown for 24 hr at 32° on YT plates or on Minimal A plates containing 0.2% glycerol and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml (6). Colonies of nonlysogenic FMA-10 were grown overnight at 37° on YT plates.

Phage strains were λvir (kindly provided by J. G. Sutcliffe) and λ*plac5* cI857 *Sam7*. Host cells and phage were plated in 2.5 ml of H soft agar (0.8% agar) over H bottom agar (1% agar) (6), and phage plaques were allowed to form overnight at 37° on lawns of QD5003(*suIII*$^+$) or at 32° on FMA-10(λcI857). Because FMA-10 is *thy*$^-$, all plates and media contained 10 μg of thymidine per ml.

Antiserum. New Zealand White rabbits were immunized with 1 mg of electrophoretically pure β-galactosidase (7, 8) in complete Freund's adjuvant (Difco). Booster injections were administered in incomplete Freund's adjuvant (Difco) 2 and 3 weeks after the initial injection, and the rabbits were bled 1 week later. Ten microliters of immune serum precipitated 10 μg of pure β-galactosidase.

The IgG fractions of rabbit pre-immune and rabbit anti-β-galactosidase immune sera were prepared by ammonium sulfate precipitation followed by DEAE-cellulose (Whatman, DE-52) chromatography (9) in 25 mM potassium phosphate, pH 7.3/1% glycerol. Fractions containing the bulk of the flow-through material were pooled, and protein was precipitated by adding ammonium sulfate to 40% saturation. The resulting pellet was resuspended in one-third the original serum volume of 25 mM potassium phosphate, pH 7.3/0.1 M NaCl/1% glycerol, and dialyzed against the same buffer. After dialysis, any residual precipitate was removed by centrifugation. IgG fractions were stored in aliquots at −70°.

Iodination of IgG. IgG fractions were radioiodinated by the method of Hunter and Greenwood (10). The 25-μl reaction mixture contained 0.5 M potassium phosphate (pH 7.5), 2 mCi of carrier-free Na^{125}I, 150 μg of IgG, and 2 μg of chloramine T. After 3 min at room temperature, 8 μg of sodium metabisulfite in 25 μl of PBS was added, followed by 200 μl of PBS containing 2% normal rabbit serum. The ^{125}I-labeled IgG was purified by chromatography on a Sephadex G-50 column equilibrated with PBS containing 2% normal rabbit serum. The ^{125}I-IgG elution fraction was diluted to 5 ml with PBS containing 10% normal rabbit serum, filtered through a sterile Millipore VC filter (0.1 μm pore size), divided into aliquots, and stored at −70°. The specific activities were 1.5×10^7 cpm per μg.

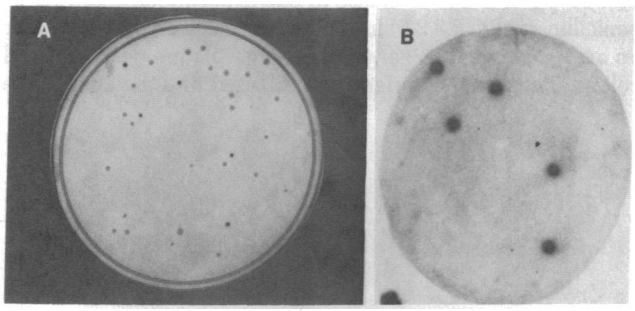

FIG. 1. Identification of *lac*$^+$ colonies by solid-phase radioimmunodetection. A mixture of RV (Δ*lac*) (λcI857) and FMA-10 (λcI857) cells was spread on a glycerol Minimal A plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside. (*A*) Colonies formed after 24 hr of growth at 32°. Due to the presence of hydrolyzed indicator substrate, the five *lac*$^+$ strain colonies appear darker than the *lac*$^-$ strain colonies. Cells within each colony on this plate were lysed by prophage induction; released antigen was adsorbed to a polyvinyl disk that had been coated with anti-β-galactosidase IgG. Immobilized antigen was labeled by incubating the polyvinyl disk with radioiodinated anti-β-galactosidase IgG. (*B*) Autoradiograph of this polyvinyl disk exposed on No Screen film for 48 hr.

RESULTS

Specificity of solid-phase ^{125}I-antibody binding

Lac$^+$ colonies growing on a minimal plate containing glycerol as the carbon source and the β-galactosidase indicator substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside develop a blue coloration while *lac*$^-$ colonies remain white. Fig. 1*A* shows such a plate bearing colonies of either FMA-10(λcI857) (*lac*$^+$) or RV(λcI857) (*lac* deletion) cells, after 24 hr of colony growth at 32°. Since no inducer of *lac* transcription was present in the plate, each cell in the five dark (*lac*$^+$) colonies contained approximately 10–20 molecules of β-galactosidase (7).

We lysed the cells within the *lac*$^+$ and *lac*$^-$ colonies by heat induction of the λcI857 prophage and adsorbed any β-galactosidase released onto a polyvinyl disk coated with anti-β-galactosidase antibodies. Fig. 1*B* shows an autoradiograph of this plastic disk after labeling with I^{125}-anti-β-galactosidase. The regions of ^{125}I-antibody binding clearly show the positions of *lac*$^+$ colonies, while the *lac*$^-$ colonies do not label.

This detection of the basal level of β-galactosidase represents essentially a full signal for this assay. If the cells had been fully induced, the labeled spots would have been larger but not more intense. Since only background labeling is observed when uninduced *lac*$^+$ colonies are screened with uncoated polyvinyl or coated with pre-immune serum IgG, the fixed, specific antibody is required. Faintly detectable amounts of antigen can be adsorbed, however, by uncoated polyvinyl exposed to lysed, fully induced *lac*$^+$ colonies in which approximately 2% of total protein is β-galactosidase.

Fig. 2*A* shows that ^{125}I-antibody binding is dependent upon *lac*$^+$ cell lysis and demonstrates an application of this solid-phase radioimmunodetection to phage plaques. This disk was applied to a plate bearing plaques of λvir phage on a lawn of

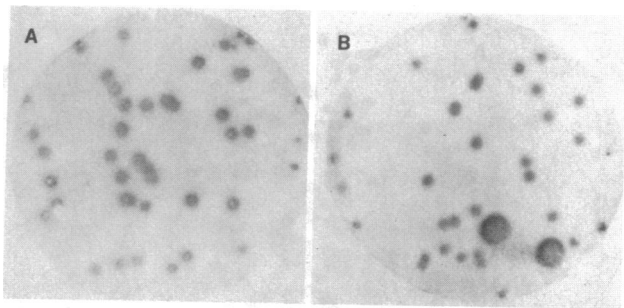

FIG. 2. Solid-phase radioimmunodetection of β-galactosidase present in phage plaques. Antigen released from host cells lysed during phage plaque formation was immobilized on a polyvinyl disk and labeled as described in the legend to Fig. 1. The autoradiographs are of disks imprinted on plates bearing (*A*) λvir plaques on a lawn of FMA-10(λcI857) cells or (*B*) λvir and λ*plac5 Sam7* plaques on QD5003 cells, exposed on No Screen film for 48 hr.

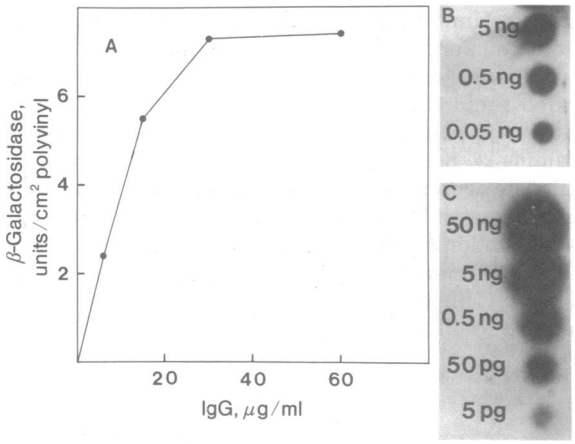

FIG. 3. Assays of IgG adsorption to polyvinyl and solid-phase radioimmunodetection of two different antigens. (*A*) Polyvinyl disks (8.25-cm) were coated with various dilutions of anti-β-galactosidase IgG in 20 mM $NaHCO_3$ (pH 9.2) for 1 min at room temperature. The coated disks were washed twice with 10 ml of cold wash buffer, and 1-cm^2 pieces cut from these disks were each incubated with 1 ml of wash buffer containing 1 mM $MgSO_4$, 500 mM 2-mercaptoethanol, and 1 μg of β-galactosidase for 4 hr at 4°. Unbound antigen was then removed by washing each 1-cm^2 piece of polyvinyl twice with 5 ml of cold wash buffer. Solid-phase-bound β-galactosidase was measured by *o*-nitrophenyl-β-D-galactoside hydrolysis (6). One unit of enzyme hydrolyzes 1 nmol of *o*-nitrophenyl-β-D-galactoside per min at 28°. (*B*) Aliquots (1-μl) of wash buffer containing the indicated amounts of β-galactosidase were applied to the surface of an agar plate. After the liquid had entered the agar, the antigen was adsorbed in the usual way to a polyvinyl disk that had been coated with anti-β-galactosidase IgG. Immobilized antigen was labeled and detected as described in the legend to Fig. 1, except autoradioagraphy was for 18 hr with an intensifying screen at −70°. (*C*) Aliquots (1-μl) of wash buffer containing the indicated amounts of *E. coli* penicillinase (EC 3.5.2.6) were used in a similar experiment. The anti-penicillinase IgG was prepared from an immune serum of a titer comparable to that of our rabbit anti-β-galactosidase immune serum.

uninduced FMA-10(λcI857). The IgG-coated polyvinyl disk detects the 10 β-galactosidase molecules per cell released as the cells lyse during the formation of the plaque but does not pick up any antigen from the unlysed lawn. The control labeling of IgG-coated polyvinyl disks placed upon λvir plaques on the *lac*⁻ strain RV(λcI857) is completely uniform and of background intensity (data not shown).

The autoradiographic image is larger than the original colony or phage plaque because the antigen diffuses in the plate. The spread from phage plaques is especially apparent since infection and lysis of host cells (embedded in 0.8% top agar) occurs over an extensive period of time. Fig. 2*B* shows the distribution of antigen released during λ*plac5 Sam7* and λvir growth on a *lac*⁺ *suIII*⁺ host, QD5003. The large exposed areas on the autoradiograph correspond to λ*plac5 Sam7* plaques and reflect the high level of β-galactosidase produced during the *I*⁻, Z⁺ *lac* phage infection. Each λ*plac5 Sam7* plaque contained on the order of 10–20 ng of β-galactosidase (assuming 10^6 cells lysed per plaque and 10^4 molecules of enzyme per cell). The smaller exposed areas on the film correspond in both size and position to λvir plaques, each of which contained approximately 0.1 ng of β-galactosidase (10^7 cells lysed per plaque, 10 molecules of enzyme per cell). The identity of the phage within each plaque was confirmed since only phage isolated from ostensible λvir plaques produced "macroplaques" on the lysogenic, nonsuppressing host RV(λcI857). Fig. 2*B* demonstrates that the enlargement of the autoradiographic image is proportional to the amount of β-galactosidase released from lysed cells, and further shows, therefore, that the antigen recognized by ^{125}I-antibody is β-galactosidase.

Selection of solid-phase assay conditions

We determined the appropriate polyvinyl coating conditions by measuring the amounts of β-galactosidase immobilized on 1-cm^2 pieces of polyvinyl cut from disks that had been incubated with various dilutions of anti-β-galactosidase IgG. The bound β-galactosidase retained enzymatic activity and was assayed by *o*-nitrophenyl-β-D-galactoside hydrolysis (7). Fig. 3*A* shows that a 1-min incubation with 30 μg of IgG per ml saturates the antibody-adsorbing capacity of an 8.25-cm diameter polyvinyl disk. In addition, the same amount of β-galactosidase is bound by polyvinyl coated for 1 min or for 5 hr with 30 μg of IgG per ml. By the same procedure, we found that 10 disks coated successively with one 10-ml solution of 60 μg of IgG per ml possess identical capacities to bind β-galactosidase. A decrease in binding was observed, however, as disks were coated successively in 10 ml of 30 μg of IgG per ml, from which we estimate that each disk binds about 20 μg of antibody.

In order to estimate the minimum amount of protein detectable using 5×10^6 cpm of ^{125}I-IgG, we applied a series of dilutions of antigen directly to the surface of a typical agar plate. The antigen then was adsorbed to an IgG-coated polyvinyl disk and labeled with ^{125}I-antibodies. Fig. 3 *B* and *C* shows that 50 pg of β-galactosidase in one experiment and 5 pg of penicillinase (EC 3.5.2.6) in another were detected, spread over an area somewhat larger than a phage plaque or a bacterial colony. Because the antigen had diffused into the agar, these

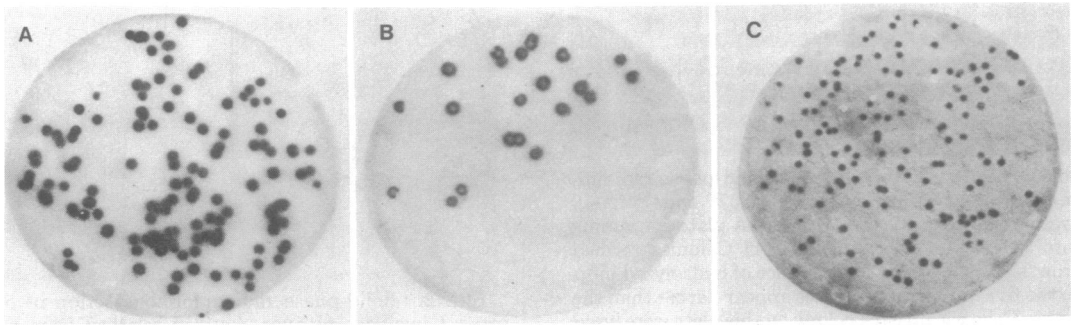

FIG. 4. Autoradiographs illustrating three methods for releasing antigens from bacterial colonies. (*A*) Colonies of FMA-10(λcI857) were grown for 24 hr at 32° on a YT plate. Cells within these colonies were lysed by prophage induction during a 2-hr incubation at 42°. (*B*) Cells within FMA-10 colonies, formed overnight at 37° on YT plates, were lysed *in situ* by applying 2 μl of 10 mM $MgSO_4$ containing 10^5 λvir to each colony on one-half of a plate and then incubating this plate for 3 hr at 37°. The other half of this plate serves as a control. (*C*) Similar colonies were lysed by a 10-min exposure to chloroform vapor in a tightly sealed glass container, as described in *Materials and Methods*.

experiments detected only a fraction of the initial sample.

The specific labeling of the immobilized antigen is maximal after an overnight incubation with ^{125}I-IgG. One-half maximal labeling is reached after 5 hr.

Alternative methods of lysing colonies

As Fig. 4 shows, comparable amounts of β-galactosidase are released from colonies by prophage induction or by direct application of λvir. Chloroform vapor also will lyse cells sufficiently to permit detection of β-galactosidase present in colonies of cells uninduced for *lac* expression (Fig. 4*C*). Colonies of RV(λcI857) (*lac* deletion) cells lysed by any of these methods did not release any material that reacted with ^{125}I-anti-β-galactosidase IgG.

Viable bacteria exist within a colony of λcI857 lysogens after a 2-hr incubation at 42°. Many of the survivors are lysogens and can be recovered, to confirm a positive response, by picking from the site of a colony. Replica plates must be used if colonies are to be treated with λvir or with chloroform vapor.

DISCUSSION

This solid-phase screening method is simple and sensitive. It detects a few picograms of protein antigen, a few molecules from each bacterial cell, using the IgG fraction from an immune serum of moderate titer. The only clear requirement for this approach is that the antigen bind at least two antibody molecules simultaneously.

Enzymatic assays suggest that about 2×10^8 molecules of β-galactosidase can bind per mm^2 of coated plastic. This is consistent with our estimate of the IgG-adsorbing capacity of the polyvinyl, 2×10^{10} molecules per mm^2, since the specific antibodies constitute only a few percent of the immune IgG fraction. Direct counting of samples of labeled plastic showed that only about 5×10^7 labeled antibodies were bound to each mm^2 of fixed antigen under the conditions described; at least 10-fold more label could bind, but at a price in terms of a higher background. The lower limit of detection presumably could be extended by using affinity-purified antibodies.

Uses for this immunological screening procedure include direct identification of clones containing specific foreign DNA segments, if they express a translation product either fortuitously or after *in vitro* genetic manipulations to that end. Furthermore, this technique provides a simple way to follow the movement of antigen on columns or on slab gels.

This two-site detection is particularly suited for the recognition of certain novel genetic constructions which are much less easily assayed by *in situ* immunoprecipitation approaches. For instance, by coating polyvinyl disks with an IgG fraction prepared from an immune serum directed against one protein and labeling the immobilized antigen with ^{125}I-antibodies directed against another protein, only hybrid polypeptide molecules, synthesized as the result of *in vitro* or *in vivo* DNA sequence rearrangement, would produce an autoradiographic response.

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