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Autoradiographic Detection of IgG and Viral Antigens

J.C. Gerdes *,***, I. McNally ***, L. Hileman *** and J.S. Burks ¹,***,***

* Veterans Administration Medical Center, Denver, CO, ** Departments of Microbiology/Immunology and Neurology, School of Medicine, University of Colorado Health Sciences Center, Denver, CO, and *** Rocky Mountain Multiple Sclerosis Center, Denver, CO, U.S.A.

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Autoradiographic methods can be used as an alternative to indirect immunofluorescence to detect viral antigen expression or the presence of IgG in tissue sections. Iodinated protein A isolated from Staphylococcus aureus detects an influx of IgG into the central nervous system of mice inoculated with the coronavirus SD. Antispecies antibody that has been iodinated detects coronavirus antigen expression for 24 days post-inoculation while it is only detectable for 10 days by immunofluorescence. A direct comparison of indirect fluorescence and autoradiographic methods indicates that the autoradiographic techniques are considerably more sensitive. This increased sensitivity is sufficient to permit the detection of viral antigen in formalin fixed paraffin embedded tissue sections.

Key words: autoradiography — viral antigen detection

Introduction

Routine methods of detecting viral antigen expression in tissue sections include indirect immunofluorescence (Coons et al., 1941), or the use of enzyme conjugated antibodies (Engvall and Perlmann, 1971). However, these methods lack sufficient sensitivity to detect latent or persistent viruses where viral expression appears to be repressed (Stevens, 1975; Haase et al., 1977). The peroxidase–antiperoxidase method (Sternberger et al., 1970), although highly sensitive, can exhibit high background staining due to endogenous peroxidase found in areas of inflammation (Weir et al., 1977). Moar et al. (1979) proposed the use of ¹²⁵I-labeled protein A isolated from Staphylococcus aureus as an alternative method to immunofluorescence for the detection of Epstein-Barr virus associated antigens expressed in human lymphoblastoid cell lines. We report here the use of ¹²⁵I-labeled protein A to detect IgG in

¹ Correspondence to: J.S. Burks, M.D., University of Colorado Health Sciences Center, Box B181, 4200 E. Ninth Avenue, Denver, CO 80262, U.S.A.

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tissue sections and a similar method using iodinated antispecies antibody to detect
viral antigen in these sections. The results indicate that iodinated protein A or
antispecies antibody can be used as a probe to detect antigens in tissue sections
followed by autoradiography. The technique is reproducible and sensitive enough to
detect antigen in formalin fixed paraffin embedded tissue sections.

Materials and Methods

Cells and virus

Virus was grown in a mouse DBT cell line originally described by Hirano et al.
(1974), and obtained from S. Stohlman, University of Southern California, Los
Angeles. Methods of virus growth and titration have been described previously
(Gerdes et al., 1981). Coronaviruses SD and SK were isolated in our laboratory from
MS patients (Burks et al., 1980). For antigen detection studies, DBT cells grown as
monolayers on 15 mm round coverslips placed in the bottom of 24-well costar tissue
culture cluster dishes were infected with coronavirus SK at low multiplicity (50–100
plaque forming units per coverslip). Infection was allowed to progress for 16–20 h,
at which time each coverslip was rinsed 3 times in phosphate-buffered saline (PBS)
and fixed in -20°C acetone for 10 min. Following fixation, the coverslips were air
dried, mounted on glass slides with coverbond (Harleco), and stored at room
temperature in sealed slide boxes containing calcium sulfate (Drie-rite).

Tissue sections

Three-week-old, male specific pathogen-free C57 B16/J mice were obtained from
Jackson Labs., Bar Harbour, ME. Antibody titers to coronavirus SD were undetect-
able by an ELISA test prior to inoculation. Mice were inoculated intracerebrally
with 7200 plaque forming units of SD grown in DBT cells in a 0.03 ml volume.
Control mice were inoculated with a homogenate of uninfected DBT cells. Mice
were sacrificed with ether, the brain and spinal column removed, and immediately
snap frozen in a dry ice ethanol bath. Tissue was stored at ~70°C prior to cryostat
sectioning. Four μm cryostat sections were fixed in acetone at ~20°C, air dried and
used immediately. For formalin fixation, anesthetized mice were perfused through
the left ventricle of the heart with a 10% neutral buffered formalin solution until
blanching of the liver occurred. Brain and spinal cord were then embedded in
paraffin and sectioned at 5 μm. Sections are de-paraffinized through xylene and
alcohol prior to addition of primary antiserum for antigen detection.

Iodination

125I-labeled protein A was obtained from Amersham Corporation, Arlington
Heights, IL (55 mCi/mg). Rabbit anti-guinea pig IgG (Cappel) was purified using
protein A Sephadex (Pharmacia) and iodinated by the iodogen method (Markwell
and Fox, 1978). One hundred microliters containing 2 mg of protein in PBS, pH 7.0,
and 1 mCi of 125I-labeled sodium iodide (Amersham, specific activity 11–17 mCi/μg)
were added to a glass tube that had been pre-treated with iodogen (25 μg/tube)
(Pierce Chemicals). Following iodination for 10 min at 4°C, the radiolabeled protein was removed from the iodo gen tube and immediately centrifuged at 200 × g for 5 min through a 1.5 ml P6DG (Bio-Rad) minicentrifuge column as described by Christopherson (1979). Duplicate samples were TCA precipitated (5%) to determine the specific activity. Iodination by this procedure yields from $10^5$ to $10^6$ cpm/µg of protein.

**Indirect tests for antigen detection**

Tissue sections were placed on acid-cleaned chrom alum dipped glass slides (15 min at 37°C, 5 g of gelatin, 0.5 g of chromium potassium sulfate 12 hydrate in 1 liter of distilled water). Air-dried coverslips or tissue sections are placed in phosphate-buffered saline, pH 7.0, for 15 min prior to reaction with primary antiserum. The primary antiserum is a guinea pig anti-coronavirus serum prepared against purified coronavirus as described previously (Gerdes et al., 1981). Fifty microliters of antiserum are added to each tissue section or monolayer that has been circled using a wax pencil and covered utilizing siliconized glass coverslips (Siliclad, Clay Adams). The antiserum is diluted in phosphate-buffered saline containing 0.4% bovine serum albumin (PBS-BSA). It is allowed to react for 30 min at room temperature in a humidified chamber. The coverslip is then gently soaked off by dipping the slide in phosphate-buffered saline and washed using two 10-min rinses, again in phosphate-buffered saline. For immunofluorescence, 50 µl of fluorescein (FITC) conjugated rabbit anti-guinea pig IgG (Miles) diluted in PBS-BSA is allowed to react for 30 min at room temperature covered by a siliconized coverslip. The coverslip is soaked off in PBS and the sections are washed twice, 10 min each in PBS. The tissue section is then covered with 50% glycerin PBS and observed for fluorescence. For autoradiographic techniques, 50,000 cpm of $^{125}$I-labeled protein A or $^{125}$I-labeled goat anti-guinea pig IgG diluted in PBS-BSA are added to the section and again covered with a siliconized coverslip and incubated at 30 min at room temperature. The sections are then washed twice 10 min each in PBS and further washed 18 h at 4°C in a beaker containing 4 liters of PBS. Following PBS washes, sections are dehydrated with 2 changes, 10 min each, of 70% ethanol and 2 changes, 10 min each of 90% ethanol and allowed to air-dry. These slides are then dipped in liquid emulsion (NTB-2 Kodak) that has been diluted 1:1 with distilled water containing 2% glycerol at 42°C. Emulsion coated slides are air dried for 10 min at room temperature and stored at 4°C in sealed light tight boxes containing Drie-rite. Following a 7 day exposure, slides were developed in Kodak D19 for 5 min, rinsed briefly in H₂O, fixed for 8 min in Kodak Rapid Fix, and finally rinsed in running water for 30 min. Monolayers were stained with 3% Giemsa in 0.15 M phosphate, pH 7.0. Tissue sections were stained using hematoxylin eosin as modified for autoradiography by Baserga and Malamud (1969).

**Results**

*Detection of viral antigen in infected cell monolayers*

DBT cells were infected with coronavirus SK and fixed with acetone 16–18 h
Fig. 1. Coronavirus SD antigenic expression in infected DBT cells as detected by indirect immunofluorescence. Upper panel: primary anti-coronavirus antiserum diluted 1:160 (410×). Lower panel: primary anti-coronavirus antiserum diluted 1:2560 (410×).
Fig. 2. Coronavirus S0 antigenic expression in infected DBT cells as detected by staphylococcal protein A autoradiography. Upper panel: exposed silver grains observed by light microscopy (left) or dark field microscopy (right) for primary anti-coronavirus antiserum dilution of 1:2560 (2000×). Lower panel: exposed silver grains observed by light microscopy (left) or dark field microscopy (right) for primary anti-coronavirus antiserum dilution of 1:80,000 (164×).
TABLE I
COMPARISON OF INDIRECT FLUORESCENT ANTIBODY (FA) AND 125I-LABELED STAPHYLOCOCCAL PROTEIN A (SAAR) METHODS FOR DETECTION OF CORONAVIRUS SD ANTIGENIC EXPRESSION IN INFECTED I7CI-1 CELLS 

|          | FA     | SAAR   |
|----------|--------|--------|
| Working dilution \(^b\) | 1/160  | 1/2560 |
| Titration endpoint \(^c\)  | 1/5120 | 1/164000 |

\(^a\) The primary antiserum was prepared in guinea pigs by inoculation of purified virus and has an end-point titer by ELISA of 1/64,000.

\(^b\) The working dilution is defined as the dilution of primary antisera producing maximal reactivity with infected cells with minimal non-specific reactivity with uninfected cells.

\(^c\) The titration endpoint is the highest dilution of primary antiserum that retains specific reactivity.

after infection. An antiserum prepared following inoculation of purified coronavirus SK into guinea pigs and having an end-point titer by ELISA of 1 : 64,000 was titered by an indirect antigen test utilizing 125I-labeled protein A or FITC-labeled anti-IgG (FA). The titration endpoint was defined as the highest dilution of this primary antiserum that retained specific reactivity. The end-point was 1 : 5120 for FA and by staphylococcal protein A autoradiography (SAAR) the end-point was 1 : 164,000 (Table I). For both methods, with increasing dilution of the primary antiserum, the same number of infected foci was detectable, but the intensity of FA staining (Fig. 1) or numbers of silver grains (SAAR, Fig. 2) decreased. Therefore, compared to the FA test using identical sera, the 125I-labeled protein A autoradiography was approximately 32 times greater in sensitivity following a 7 day exposure time. A working dilution was also determined from these experiments (Table I) and is defined as a dilution of primary antiserum producing maximal reactivity with infected cells and minimal non-specific reactivity with uninfected cells. 125I-labeled protein A alone revealed no reaction on infected cells.

Detection of silver grains was greatly enhanced utilizing dark field microscopy (see Fig. 2), since silver grains are readily detectable even overdarkly stained nuclei. In addition, background levels become readily apparent and the cells can be screened rapidly at lower power. In order to determine the optimum concentration of protein A required to detect viral antigen, 10-fold dilutions of the 125I-labeled protein A was reacted with infected cells pretreated with a working dilution (1 : 2560) of the guinea pig anti-coronavirus antiserum. Maximal grain counts with a minimum of background was obtained utilizing 50,000 cpm per coverslip.

The detection of IgG and coronavirus antigenic expression following intracerebral inoculation of C57 B16/J mice
Coronavirus SD produces a subacute demyelinating disease in C57 B16/J mice following intracerebral inoculation. Focal, paraventricular demyelination is observed from days 6 through 24 in the spinal cord of inoculated animals. Infectious virus is only detected for the first 10 days following inoculation. For investigation of viral
Fig. 3. Background level of exposed silver grains observed by light microscopy (upper panel) or dark field microscopy (lower panel) in the temporal cortex of an infected mouse 4 days post-infection. This section was reacted with preimmune guinea pig IgG, then exposed for 7 days prior to development. Similar background levels are obtained on uninfected tissue reacted with guinea pig anti-viral antisera and $^{125}$I-labeled rabbit anti-guinea pig IgG or sections of brain or spinal cord reacted with $^{125}$I-labeled protein A (410×).
Fig. 4. Level of IgG and coronavirus SD antigen expression is an area of meningomyelitis observed in the spinal cord 2 days following IC inoculation of a C57 B16/J mouse. Upper panels: IgG is not detected by $^{125}$I-labeled protein A (410×, light microscopy left, dark field right). Lower panels: viral antigen is detected in the area of meningomyelitis using $^{125}$I-labeled antispecies antibody (410×, light microscopy left, dark field right).
Fig. 5. Level of IgG and coronavirus SD antigen expression in a focal lesion observed in the spinal cord 19 days following IC inoculation of a C57 B16/J mouse. Upper panels: IgG is readily detected throughout the lesion by $^{125}$I-labeled protein A (410×, light microscopy left, dark field right). Lower panels: viral antigen is expressed in a serial section reacted with anti-coronavirus antiserum and $^{125}$I-labeled antispecies antibody (410×, light microscopy left, dark field right).
Fig. 6. Autoradiographic detection of coronavirus antigen expression in formalin fixed paraffin embedded tissue. Viral antigen is detected in neurons of the temporal cortex (410×, light microscopy upper, dark field lower).
antigen expression, 2 coronavirus SD infected mice and 1 control DBT cell homog-
enate inoculated mouse were sacrificed daily for 12 days following inoculation. After
day 12, mice were sacrificed on days 15, 19, 22, 24, and 30. Serial cryostat sections of
brain and 4 different cross sectional areas of the spinal cord were investigated by
indirect fluorescence and autoradiography. By immunofluorescence, viral antigen
expression was detectable between days 3 and 10 post-inoculation. For autoradiog-
raphy, tissue sections were reacted with primary antiserum (1:2560 dilution) and
then with $^{125}\text{I}$-labeled rabbit anti-guinea pig IgG. Serial sections were reacted with
either preimmune antiserum and $^{125}\text{I}$-labeled anti-guinea pig IgG or $^{125}\text{I}$-labeled
protein A. As is shown in Fig. 3, uninoculated mice had a low background when
reacted with $^{125}\text{I}$-labeled protein A. A similar background is also observed for
sections obtained from infected mice and reacted with preimmune guinea pig
antigen and $^{125}\text{I}$-labeled anti-guinea pig IgG. On days 1 through 5 post-inoculation,
inflammatory areas remained negative when reacted with $^{125}\text{I}$-labeled protein A,
which should detect any IgG in the tissue (Fig. 4). However, these areas contained
abundant viral antigen detected by autoradiography following the indirect test using
labeled antispecies antibody. On days 6 through 24, however, focal lesions contain-
ing viral antigen also contained elevated levels of IgG as detectable with $^{125}\text{I}$-labeled
protein A (Fig. 5). In summary, viral antigen expression was detectable by im-
munofluorescence only for the first 10 days following inoculation while it was
detectable using autoradiographic methods until day 24 post-inoculation. A more
detailed description of the pathology associated with viral antigen expression will be
published elsewhere.

Detection of coronavirus antigenic expression in formalin fixed paraffin embedded
tissue

As a further measure of the sensitivity of the autoradiographic methods, formalin
fixed paraffin embedded tissue sections were obtained from mice 4 days post-inocu-
lation. Day 4 post-inoculation is the peak of virus replication in the brain. The
indirect FA method failed to detect antigen in these sections. However, using the
indirect autoradiographic technique coronavirus antigen was found, particularly in
neurons of the temporal cortex (Fig. 6). Similar studies of mice sacrificed on day 6
post-infection failed to detect viral antigen. Therefore, the sensitivity of the autoradi-
graphic technique is greatly reduced for paraffin embedded tissue, but it can
detect a high level of antigenic expression. Preliminary experiments suggest that
trypsin treatment, such as that utilized for immunofluorescence (Swoveland and
Johnson, 1979), does improve sensitivity of autoradiographic techniques on em-
bedded tissue.

Discussion

The method of autoradiographic antigen detection as originally proposed by
Moar et al. (1979) is a highly sensitive alternative to immunofluorescence. We have
compared the sensitivity of this method with immunofluorescence by 3 different
criteria: (a) the primary anti-coronavirus antiserum can be diluted 32-fold greater and retain specific reactivity for SAAR relative to the titration end-point of FA; (b) following intracerebral inoculation, viral antigen expression is detectable for 10 days utilizing FA, but 24 days post-inoculation by autoradiography; (c) formalin fixed, paraffin embedded tissue was negative by FA on day 4 post-inoculation, but detectable using autoradiography.

In addition to increased sensitivity, autoradiographic methods are more readily correlated with histopathology since the sections can be stained by routine hematoxylin and eosin. The autoradiograph is a permanent record, and by counting silver grains a more quantitative analysis of the degree of expression can be obtained. Finally, the interspecies reactivity of staphylococcal protein A for the Fc receptor of IgG (Cronvall et al., 1970) makes this method preferable when attempting to detect antibody from different species. Interspecies activity also allows for the detection of IgG in tissue sections.

In conclusion, autoradiographic antigen detection is a highly sensitive method that should find broad application for the detection of low level antigen expression.

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