Comparative Study of $^{125}$I- and $[^3]$HAcetate-Labeled Antibodies in Detecting Iridescent Viruses

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Radioimmunoassays for detecting cell-associated or released virus are described using either $^{125}$I- or $[^3]$Hacetate-labeled antibodies. In the first assay system, antigen-antibody complexes were separated from free antibody by centrifugation. Sensitivities of 0.1 μg of iridescent virus could be achieved with either $^{125}$I- or $[^3]$Hacetate-labeled antibody. In the second assay, the antigen was fixed to cover-slip cell cultures, and then reacted with labeled antibody, unbound radioactivity being removed by repeated washing. Nonspecific binding with this method was 0.5 to 1% of the total radioactivity added and sensitivities of 0.1 or 10 μg were achieved with $^{125}$I and $[^3]$Hacetate, respectively. Immunoglobulins were labeled at the rate of 1 in 300 for $^{125}$I and 1 in 200 with $[^3]$Hacetate although there was a 400-fold greater isotopic abundance of $^{125}$I relative to $[^3]$H. The possibility of preparing labeled protein of high specific activity using carrier-free [2-$^3$H]iodoacetic acid is discussed.

Iridescent viruses are large (130 nm) icosahedral particles containing double-stranded deoxyribonucleic acid as their genetic material. Unlike most deoxyribonucleic acid viruses, these viruses replicate in the cytoplasm and require a polymerase to synthesize viral messenger ribonucleic acid (10). Studies of iridescent viruses in insect larvae and in tissue culture have revealed that replication is drastically reduced at temperatures only slightly above the optimum of 21 C (1). The mechanism behind this phenomenon is not known.

The methods available for assaying the growth of iridescent or other insect viruses are limited. It is not yet possible to plaque insect viruses, and virus growth is not always accompanied by cytopathic effect (1). Fluorescent antibody staining has been virtually the only assay method available to insect virologists. The method, however, is tedious and difficult to quantitate. Moreover the problems of nonspecific fluorescence are considerable.

We have developed radioimmunological methods which are rapid and quantitative and can be used to assay cell-associated or released virus. Antibodies were labeled with [2-$^3$H]iodoacetic acid using a procedure recently developed in this laboratory (7, 12; A. J. Parkinson and J. Kalmakoff, manuscript in preparation), and by the chloramine-T method for $^{125}$I (5). This paper describes the results of comparative studies of $[^3]$Hacetate- and $^{125}$I-labeled antibody using Costelytra zealandica iridescent virus as antigen (6).

MATERIALS AND METHODS

Tissue culture of mosquito cells. Monolayers of Aedes aegypti (line of Singh (13) were trypsinized with Rinaldini solution, cells were diluted to 10$^6$/ml with Mitsuhashi-Maramoroch medium (11), and 1-ml amounts were dispensed into screw-cap bottles containing 13-mm-diameter cover slips. The cultures were incubated at 28 C until complete monolayers had formed.

Preparation of antigen. Iridescent virus (CaIV) from the coleopterous host C. zealandica was purified from laboratory-infected larvae by differential centrifugation and banding in sucrose gradients. Virus concentrations were determined spectrophotometrically using an absorbance at 260 nm of 18.2 cm$^2$/mg of virus (8).

Preparation of immunoglobulin. Antiserum to CaIV was prepared in rabbits by intramuscular injection of virus emulsified with Freund complete adjuvant (6). Rabbit immunoglobulins were purified by ammonium sulfate fractionation (15). Preimmune rabbit immunoglobulin was prepared using serum obtained from unimmunized rabbits.

Labeling of immunoglobulin. $^{125}$I-labeled immunoglobulin was prepared by the chloramine-T method of Hunter and Greenwood (6). The specific activity of the $^{125}$I preparation (purchased from the Radiochemical Centre, Amersham, England) was 14 mCi/μg. The efficiency of counting $^{125}$I in a Packard gamma counter or by liquid scintillation was 60%.

$[^3]$Hacetate-labeled antibody was prepared by the following procedure. A 200-μCi amount of [2-$^3$H]iodo-
acetic acid (purchased from the Radiochemical Centre; 0.52 mCi/mg) in 0.2 ml of distilled water was added to 1 to 2 mg of purified rabbit immunoglobulin in 1.0 ml of phosphate-buffered saline (PBS). After addition of 0.7 ml of 0.1 M Na₂HPO₄ (pH 9.0), the pH was adjusted to 9.8 with 0.04 M NaOH. The mixture was then incubated in the dark for 30 min at 56 C and the reaction was stopped by layering on a Sephadex G-50 column (1.5 by 30 cm). The labeled immunoglobulin was eluted with PBS and recovered by ammonium sulfate precipitation. The efficiency of counting ³H-labeled proteins was 20% as determined by the internal standardization or the external standard channels ratio method (9).

**Labeling of virus.** The procedure for labeling CsIV with [²⁻¹H]iodoacetic acid or ¹²⁵I was identical to that for labeling immunoglobulins except that labeled virus was not ammonium sulfate precipitated.

¹²⁵I-labeled virus in 100-μl volumes was layered onto cover-slip monolayers of A. aegypti cells. The virus was dried for 3 h at 37 C, acetone-fixed, and incubated under conditions used for cell-associated virus assay except that 100 μl of PBS was used instead of labeled antibody.

**Biological activity of labeled antibody.** The biological activity of each labeled antibody preparation was determined from the percentage of radioactivity bound to viral antigen. The reactions were carried out in 1.0-ml volumes in polycarbonate tubes (18 by 75 mm) at 37 C for 30 min. The iridescent virus used in this study has a sedimentation coefficient of 2.100 S (8). This enables labeled antigen-antibody complexes to be separated from unbound antibody by centrifugation for 20 min at 40,000 x g. The percentage of radioactivity bound to virus was calculated by comparison with controls containing no virus.

**Centrifugation assay of virus.** Polycarbonate centrifuge tubes (18 by 75 mm; purchased from Sorvall Corp.) were coated with 1.0% bovine serum albumin (BSA) overnight at 20 C; the fluid was then discarded and the tubes were dried. Prior to using, immunoglobulin was centrifuged at 40,000 x g for 20 min to remove aggregates formed during storage. The supernatant fluid was diluted to give a working preparation of 2 x 10⁶ counts per min per ml.

A 4-μg amount of [³H]acetate antibody or 0.04 μg of [¹²⁵I] antibody were added to virus dilutions containing 0.5% BSA in PBS (pH 7.3), and, after incubation for 20 min at 37 C, virus-antibody complexes were separated from other reactants by centrifugation at 40,000 x g for 5 min as outlined in Fig. 1. Solubilization of the pellet with 2.0% sodium dodecyl sulfate at 56 C for 30 min and counting by liquid scintillation enabled the direct comparison of [¹²⁵I]- with [³H]acetate-labeled antibody. [¹²⁵I] radioactivity was also counted by direct gamma scintillation.

**Cell-associated virus assay.** Samples (100 uliters) of 10-fold serial dilutions of CsIV were added to 7-day-old cover-slip cultures of A. aegypti previously washed two times with PBS (pH 7.3). The virus was dried onto the cells at 37 C for 3 h and acetone-fixed. Samples (100 μl) containing 1 μg of [¹²⁵I] antibody or 20 μg of [³H]acetate antibody in PBS (pH 8.0) were added to the fixed monolayers and incubated at room temperature for 1 h under humid conditions. The cover slips were washed four times with PBS (pH 8.0) to remove bound radioactivity and placed into scintillation vials. [³H]acetate samples were counted in water-miscible scintillation fluid, and [¹²⁵I] samples were counted by the direct gamma method.

**RESULTS**

**Assay of virus by centrifugation.** When 10 μg of either [¹²⁵I]-labeled (specific activity 100,000 disintegrations per min per μg) or [³H]acetate-labeled antibody (specific activity 2,100 disintegrations per min per μg) was reacted with varying amounts of viral antigen,
34% of the radioactivity was bound to virus, suggesting the remainder is attached to proteins other than antibody to CzIV; 25 μg was enough virus to bind all the labeled antibody in the system (Fig. 2).

A comparison of the ability of 125I- and [3H]acetate-labeled antibody to detect microgram amounts of viral antigen is presented in Fig. 3. Both preparations were capable of detecting 0.1 μg of virus by this method. Controls using preimmune immunoglobulin showed no detectable reaction at any of the virus concentrations (Fig. 3).

Efficiency of fixing virus onto monolayers. CzIV particles were labeled with 125I or [3H]acetate as described in Materials and Methods. The purity and physical condition of labeled virus was compared by centrifugation in sucrose gradients with unlabeled virus as a marker. There was no detectable physical damage to the virus by these labeling conditions. 125I-labeled virus had a specific activity of 140,000 disintegrations per min per μg and [3H]acetate-labeled virus had a specific activity of 900 disintegrations per min per μg.

![Fig. 2. The biological activity of labeled immunoglobulin. Each assay contained 10 μg of either [3H]acetate-labeled antibody (2 × 10⁶ counts/min) (●) or 125I-labeled antibody (6 × 10⁶ counts/min) (○) and iridescent virus (CzIV) in a total volume of 1.0 ml. The radioactivity bound to the antigen was obtained by subtracting supernatant counts from the total input radioactivity.](image-url1)

![Fig. 3. A comparison of the sensitivity of [3H]acetate- and 125I-labeled antibody for the detection of viral antigen. (A) 125I-labeled antibody (0.04 μg) (approximately 2,000 counts/min) was incubated with virus, and then unbound antibody was separated by centrifugation (see Fig. 1). The percentage of radioactivity in the pellet was measured either by direct gamma scintillation (●) or solubilizing for liquid scintillation (○). (B) [3H]acetate-labeled immunoglobulin (4.0 μg) (2,000 counts/min) was incubated as above, and the bound antibody was counted by liquid scintillation (●). 125I- and 125I-labeled preimmune antibody were used as controls (△).](image-url2)

Using 125I-labeled CzIV, we found that 60 to 70% of virus is fixed to the cells (Table 1). The figure for 0.01 μg of virus is not reliable as the radioactivity at this dilution was very low.
Cell-associated virus assay. The ability of $^{125}$I- and $[^3H]$acetate-labeled antibody to specifically bind cell-associated virus is illustrated in Fig. 4. A 0.1-µg amount of virus can be detected using $^{125}$I-labeled antibody and 10 µg can be detected with $[^3H]$acetate-labeled antibody.

Fig. 4A shows that a semilogarithmic relationship exists between virus present and $^{125}$I antibody bound in the 0.1 to 100 µg virus range. It can therefore be calculated that a 10-fold increase in virus results in a 3,600 counts/min increment in radioactivity bound. Virus growth in tissue culture can thus be quantitated in terms of virus increment rather than increase in bound radioactivity using the relationship $V = \text{antilog } R/K$, where $V$ is the factor by which virus has increased; $R$ is the increase in radioactivity bound; and $K$ is the slope of the standardization curve, in this case, $K = 3,600$ counts per min per log increment virus.

**TABLE 1. Efficiency of binding of $^{125}$I-labeled virus to A. aegypti cells**

| $^{125}$I-labeled virus (µg) | Input radioactivity bound (%) |
|-----------------------------|-------------------------------|
| 100                         | 69                            |
| 10                          | 56                            |
| 1                           | 61                            |
| 0.1                         | 74                            |
| 0.01                        | 100                           |

*a $^{125}$I-labeled virus in 100-µliter volumes was layered onto cover-slip monolayers of A. aegypti cells as described in Materials and Methods and incubated under conditions used for cell-associated virus assay except that 100 µliters of PBS was used instead of labeled antibody.

**Fig. 4. Assay of cell-associated virus using $^{125}$I- and $[^3H]$acetate-labeled immunoglobulins.** Samples (100 µliters) containing 20 µg of $[^3H]$acetate-labeled (1,400 disintegrations per min per µg) or 1 µg of $^{125}$I-labeled immunoglobulin (100,000 disintegrations per min per µg) were added to virus fixed onto cover-slip cultures (see Table 1). After incubation for 1 h at 37 C, the cover slips were washed four times with PBS to remove unbound radioactivity. (A) $^{125}$I radioactivity was determined by gamma scintillation; (B) $[^3H]$ radioactivity was determined by solubilizing for liquid scintillation. Anti-CaIV immunoglobulin (●); preimmune rabbit immunoglobulin (○).

**DISCUSSION**

The critical step common to all radioimmunoassays is the separation of antigen-antibody complexes from unreacted labeled reagents. The large sedimentation coefficient of the iridescent viruses and the use of a small reaction volume enables antigen-antibody complexes to be selectively removed in the centrifugation assay (Fig. 1). The very high efficiency of this process is reflected in the extremely low levels of nonspecific binding (0.1%) observed with preimmune immunoglobulin (Fig. 3). In the assay of cell-associated virus, washing of cover slips prior to measuring radioactivity was essential, and the level of nonspecific binding of antibody was approximately 1 and 0.5% for $^{125}$I- and $[^3H]$labeled immunoglobulin, respectively.

Different relative sensitivities were obtained with $^{125}$I- or $[^3H]$acetate-labeled antibodies depending on the type of assay method used. The centrifugation assay (Fig. 3) shows that using equal levels of either $^{125}$I or $[^3H]$acetate radioactivity results in a sensitivity of 0.1 µg of virus. Assuming a molecular weight of $5.51 \times 10^8$ (8), this represents $10^8$ iridescent virus particles. Thus, despite the almost 50-fold difference in the specific activities of $[^3H]$acetate (2,100 disintegrations per min per µg) and $^{125}$I (100,000 disintegrations per min per µg), the sensitivity remained the same. At the above specific activities, the frequency of radioactively labeled immunoglobulins is 1 molecule in 200 and 1 molecule in 300 for $[^3H]$acetate and $^{125}$I preparations, respectively. Theoretically, with equal microgram amounts, slightly better results should have been obtained in the $[^3H]$acetate system. This, however, is not possible due to the 72-fold difference in isotopic half life.

In the cell-associated virus assay, $^{125}$I appears to have a definite advantage over $[^3H]$acetate as the labeling nuclide. Whereas the sensitivity is 0.1 µg of virus (10⁴ particles) using $^{125}$I (Fig. 4A), as much as 10 µg (10¹⁰ particles) was required using $[^3H]$labeled antibody (Fig. 4B). It is not possible to directly compare the cell-associated virus assay with the centrifugation method since it is not known what effect acetone fixation has on the virus, and the cell-associated virus assay is essentially a solid-phase system with different kinetics. It is probable that the requirement for higher concentrations of antibodies in the $[^3H]$acetate assay.
results in undesirable competition by unlabeled antibodies.

Under our experimental conditions, the biological activity of immunoglobulin was not affected by labeling with iodoacetic acid (Fig. 2; reference 12). More recent trials have shown that the maximum specific activity that can be obtained without any loss in biological activity was at 70 C and pH 9.8. Furthermore, labeling with iodoacetic acid does not alter the specificity of the antibody (7).

Use of [2-3H]iodoacetic acid at higher concentrations did not result in better labeling (S. Bilimoria, unpublished data). The pH of the reaction mixture drops to well below the optimum of 9.8 at these concentrations, and, owing to the small reaction volume, adjustment with alkali was not possible under the experimental conditions used. According to our calculations, the isotopic abundance of the [3H]acetate preparation used was 0.25%, i.e., only 1 in 400 iodoacetic molecules in the preparation was radioactive. If carrier-free [3H]iodoacetic acid was used, a much higher specific activity of labeled protein could be obtained. It is possible to attach up to two atoms of 125I per tyrosine residue of an immunoglobulin molecule (4). On the other hand, iodoacetic is capable of binding four different amino acid residues (3, 14). This is reflected in the finding that the frequency of labeled protein is slightly better with [3H]acetate in spite of the lower isotopic abundance of the [3H]acetate preparation. Therefore, it is not difficult to envisage proteins labeled at the rate of at least one nuclide per molecule using carrier-free [3H]acetate. The specific activity of such a preparation would be about 420,000 disintegrations per min per µg. This indicates that labeling proteins with [3H]acetate is a feasible technique, giving rise to preparations with high specific and biological activities providing that a method can be developed to produce carrier-free [2-3H]iodoacetic acid.

Although an infectivity-to-particle ratio was not carried out, a ratio of 1:80 was determined for another iridescent virus (1); if we assume a similar ratio, the sensitivity of the radioimmunoassays described in this paper is 10^4 infectious particles. This compares favorably with the sensitivity of 10^6 plaque-forming units per ml obtained by Dalrymple et al. (2) using 14C- and 3H-labeled Sindbis virus in a precipitation competition essay. Work on the application of the assays described in this paper to study the growth of iridescent viruses in cultured mosquito cells is now in progress.

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