NOTES

Properties of Feline Leukemia Virus

II. In Vitro Labeling of the Polypeptides¹

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Radioactive labeling of proteins from guanidine-hydrochloride disrupted feline leukemia virus was done in vitro by attaching [³H]methyl groups to protein amino groups with reductive alkylation. When in vitro labeled disrupted virus was analyzed by gel filtration in the presence of 6 M guanidine-hydrochloride the expected six major and one minor protein peaks were detected. When the same preparation was analyzed by immunodiffusion, the three antigens tested were found to have retained their antigenic activity.

The method of gel filtration in 6 M guanidine-hydrochloride (GuHCl) (1) was found to be an effective method for separating polypeptides from avian (2) and mammalian (4) oncornaviruses including feline leukemia virus (FeLV) (3). However, the assay of fractions for optical density is complicated by the presence of 2-mercaptoethanol or oxidized dithiothreitol (DTT) and requires use of a turbidometric method (1) or dialysis of individual fractions (2). Use of radioactive marker virus (2-4) eliminates these time consuming problems and permits rapid assay and detection of peak fractions. However, preparation of radioactive virus labeled in vivo is expensive, even in systems that produce large amounts of virus (3). In an effort to reduce operating expenses, a less expensive in vitro labeling method was sought. Iodination of disrupted virus was attempted and freshly prepared samples have gel chromatography profiles identical to those of in vivo labeled virus. However, after 1 to 2 months of storage some changes occur and significant radioactivity elutes in the low molecular weight region following the smallest protein (D. Graves and L. Velicer, unpublished data). In addition, labeling with radioactive iodine yields a product with a relatively short half-life and involves difficulties associated with gamma radiation. Rice and Means (5) have reported that reduc-

tive alkylation was found to be a rapid, inexpensive, and gentle procedure for introducing ³H or ¹⁴C label into most proteins including whole virus and some enzymes. We have extended the use of this method to our work with feline leukemia virus and found that in vitro labeled disrupted virus has the same peaks of radioactivity upon gel filtration analysis and the three antigens tested retain their immunological activity.

The source of virus and methods for radioactive labeling and purification of virus were as previously described (3). FeLV proteins were labeled by disrupting 1 mg of whole purified virus in approximately 0.75 ml of 8 M GuHCl dissociating buffer exactly as was done when preparing virus for gel filtration analysis (3) and then following the reductive alkylation method described by Rice and Means (5). The solubilized virus was dialyzed overnight against several changes of 0.2 M sodium borate buffer, pH 9.0, and adjusted to exactly 1 ml volume with the same buffer. To this solution, held on ice, 100 μ liters of 0.04 M [³H]formaldehyde (NET-099, 100 mCi/mmol, New England Nuclear Corp., Boston, Mass.) was added. This was followed in 30-s intervals by four 20- μ liter sequential additions of sodium borohydride (5 mg/ml, prepared fresh each time). To ensure complete reduction of the formaldehyde an additional 100 μ liters of sodium borohydride solution was added after 1 min. Soluble isotope was removed from the viral proteins by extensive dialysis against TNE buffer (0.01 M Tris, pH 7.4, 0.1 M NaCl, 0.001 M EDTA) over a

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period of 3 to 4 days. At this stage some of the FeLV proteins became insoluble and care had to be taken to stir the sample thoroughly whenever it was transferred in order to ensure representative sampling. The disrupted virus labeled in vitro with ^3H was mixed with whole virus labeled in vivo with ^{14}C (3) and analyzed by gel filtration using the small scale method (3) with the following modification. After the pelleted ^{14}C -labeled virus was solubilized in 8 M GuHCl dissociating buffer for several minutes the desired volume of disrupted in vitro labeled virus was added (usually 25 μl or less) along with dry GuHCl and concentrated DTT solution to bring the added liquid up to optimal dissociating conditions (8 M GuHCl and 0.05 M DTT). The disrupted and in vitro labeled virus was also analyzed for immunological activity after dialysis against TNE buffer. The methods for immunodiffusion analysis and preparation of sera against proteins p30, p15, and p11 were already described (3).

The results of gel filtration analysis (Fig. 1) clearly demonstrate that all the proteins labeled in vivo are also labeled in vitro. The relative amounts of isotope in p11 and p10 are reversed, however, but the reason for this difference is unknown. The apparent difference in the relative amounts of gp70 labeled by the two methods in comparison to gp ≥ 100 is related to a problem with the individual preparation of

^{14}C marker virus used in this experiment and not the method of in vitro labeling. In fact the amount of ^3H in in vitro labeled gp70 in relation to gp ≥ 100 is identical to that obtained in our earlier work (3) and the reasons for a difference in the profile of this particular preparation of ^{14}C -labeled marker virus are unknown.

The immunodiffusion data (Fig. 2) clearly show that the same three antigens renatured from GuHCl (3) retained their immunological activity despite successive treatment with 8 M GuHCl, borate buffer (pH 9.0), and reductive alkylation that are involved in the disruption and in vitro labeling of virus. When a quantitative comparison of immunological activity was made between alkylated and nonalkylated proteins by twofold serial dilution and immunodiffusion analysis, only a slight reduction was seen in the titers of p30, p15, and p11 (L. Velicer, preliminary unpublished data). In each case the precipitin lines of the alkylated proteins were either less intense than heavy control precipitin lines at the last positive dilution or they ended one dilution lower when the last positive control line was faint. These data suggest that the reduction in titer is less than 50%. However, this change is within the experimental error of the assay and a more sensitive procedure will be needed to eventually quantitate the small reduction in immunological activity.

The ability to identify the typical polypeptide

FIG. 1. Gel filtration analysis of FeLV polypeptides from a mixture of virus labeled with [^{14}C]amino acids in vivo and [^3H]formaldehyde in vitro. Purified ^{14}C -labeled whole virus was pelleted and dissociated in 8 M GuHCl dissociating buffer (3). As soon as the pellet was solubilized the desired volume of ^3H -labeled disrupted virus was added along with the amount of GuHCl and DDT needed to maintain the dissociating conditions as described in the text. After dissociation of the mixture was completed the sample was loaded on a small scale 6% agarose column (3) and eluted with 6 M GuHCl in the presence of 0.01 M DTT at a rate of 0.18 ml/h. Eluted fractions were assayed for radioactivity as previously described (3).

FIG. 2. Immunodiffusion analysis of antigens in GuHCl disrupted and *in vitro* labeled FeLV. Outer wells A, B, and C: antisera prepared against rechromatographed proteins p30, p15, and p11, respectively (3). Center well 1: feline leukemia virus that had been disrupted in 8 M GuHCl dissociating buffer (3), *in vitro* labeled with [^3H]formaldehyde by reductive alkylation as described in the text, and dialyzed against TNE buffer (3).

peaks from *in vitro* labeled virus after gel filtration analysis suggests that the method might be useful for preparing inexpensive marker virus. The labeling method gave a preparation with a specific activity of 2.5×10^6 counts/min of ^3H per mg of protein. The cost of this tritium labeled virus is about 4% of that for *in vivo* [^{14}C]amino acid-labeled marker virus routinely used in these studies (Fig. 1) (3). Furthermore, when a direct comparison is made between the cost of the same isotope incorporated *in vivo* and *in vitro*, the savings will be as great or greater for both ^3H and ^{14}C (L. Velicer, unpublished data). In addition to a 25-fold or greater reduction in the cost of marker virus, one can use purified virus that is already available in the laboratory or from commercial or other sources and avoid the time consuming *in vivo* labeling and purification of radioactive virus.

Although our interest in this labeling method resulted from a need for economic marker virus, it is apparent that the method might also be useful for labeling rechromatographed individual polypeptides (3), especially when a preparation with a long half-life is needed. It is important in this respect that the immunological activity of three proteins is not extensively destroyed by the entire procedure and immunological analysis can still be done when needed. Although the presence of protein molecules that entirely escaped methylation might explain the retained antigenicity, a calculation of methyl groups added per protein molecule suggests that this is not the case. Using data on specific

activity of [^3H]formaldehyde (100 mCi/mmol), incorporation of isotope (2.5×10^6 counts per min per mg of protein), and the 2.5 to 5% efficiency of tritium counting under the filter disk assay conditions used (3), it was determined that 10 to 20 [^3H]methyl groups were added per polypeptide assuming an average molecular weight of 42,000 calculated from data for seven polypeptides obtained in presence of GuHCl (3). According to the Poisson distribution, each protein molecule would be expected to have at least one and probably several [^3H]methyl groups, suggesting that a minimal addition does not destroy immunological activity.

Although *in vitro* labeling with ^3H has several distinct advantages over iodination there are several limitations also. Use of ^3H and ^{14}C does not permit the rapid simple assay in a gamma counter with minimal sample preparation, as is possible with iodination. Secondly, it is probably not possible to achieve the very high specific activities attained by iodination. Nevertheless, the method will be useful in many experiments when a long half-life is desirable, when extremely high specific activities are not needed, or when samples must still be prepared for scintillation counting because a second isotope is used.

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