Nonspecific Adsorption of Proteins to Microplates

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Received for publication 22 November 1972

When a solution of purified adenovirus hexon proteins was diluted in polystyrene microplates without carrier proteins in the diluent, a higher rate of hexon adsorption to the microplates at higher dilutions took place. Addition of gelatin (0.01%), bovine serum albumin (0.01%), or calf serum (0.2%, vol/vol) to the diluent effectively prevented this adsorption. The adsorption to the microplates of adenovirions and measles virions was also prevented by the carrier proteins in the diluent. Certain additional features which distinguish the microtechnique of Takatsy from conventional macromethods are also discussed.

Since the introduction of the microtitration system by Takatsy in 1950 and the modification by Sever (12) in 1962, the method has become widely used in many laboratories all over the world. It has many great advantages in saving both time and money. At the same time, however, it has a disadvantage of greater variation of the titers obtained because the volume handled is smaller than that handled in the conventional tube method. In addition, there are some other features which are characteristic of the micromethod and may have some importance in performing the technique. These will be discussed later.

A good technique to determine whether a twofold serial dilution is correctly done is to use a solution with high radioactivity as a sample to be diluted and measure the radioactivity of each dilution (11). In this report, radioisotope-labeled purified proteins were used and their adsorption to microplates was quantitatively measured. The importance of adding carrier proteins to the diluent for "dilution" methods (not only in the microtitration method but also in the conventional macromethod) was confirmed.

MATERIALS AND METHODS

Microtechnique. The dilution was done with great care with polystyrene microplates (IS-MRC-96; Lindbro Chemical Co., New Haven, Conn.) and 25 μliter-microdiluters (Cooke Engineering Co., Alexandria, Va.). Four different diluents were used: phosphate-buffered saline (PBS; 0.07 M phosphate, 0.14 M NaCl, pH 7.4) without proteins; PBS plus 0.01% gelatin; PBS plus 0.01% bovine serum albumin (BSA); and PBS plus 0.2% (vol/vol) calf serum (CS). When diluted samples were pooled and transferred to polyethylene scintillation vials, a Biopette (Schwarz/Mann, Orangeburg, N.Y.) with a polyethylene tip was used to minimize the adsorption of the sample to the pipette.

Preparation of radioisotope-labeled proteins: adenovirus type 2 (Ad 2) virion. Monolayers of KB cells were prepared in 300-ml roller bottles with Eagle minimal essential medium (MEM) plus 5% unheated fetal calf serum (FCS). The cells were infected with Ad 2 virus at a multiplicity of infection (MOI) of 0.1 to 1.0, and maintained in Eagle MEM plus 2% FCS. One day later, the medium was replaced with Eagle MEM containing H-thymidine (1 μCi/ml) and 2% FCS. Three days after infection, cells were harvested and stored at -20 C. After thawing, cells were treated with 0.2% sodium deoxycholate at pH 9.0 (6). The supernatant fluid was centrifuged in a discontinuous CsCl gradient as described by Norrby et al. (8). The virion band was harvested by piercing the bottom of the tube.

Ad 3 hexon. KB cell monolayers in roller bottles were infected with Ad 3 virus at an MOI of 10 to 100, and maintained in Eagle MEM plus 2% FCS. One day later, the medium was replaced with Eagle MEM containing "cold" L-threonine (1 μCi/ml), "cold" L-threonine (4 μg/ml), and 2% FCS. Three days after infection the cells were harvested. Virions were prepared in the same way as mentioned above. The soluble fraction of the discontinuous CsCl gradient centrifugation was dialyzed against 0.04 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0), 0.1 M NaCl, and then chromatographed in a diethylaminoethyl-Sephadex A25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column with an NaCl gradient elution as described by Norrby (7). The hexon fraction with highest radioactivity was used.

Measles virion. Monolayers of Vero cells in roller
bottles were infected with measles virus at an MOI of 0.1 to 0.01 and maintained with Eagle MEM plus 3H-L-threonine (10 µCi/ml), "cold" L-threonine (3 µg/ml), and 2% FCS. Three to 4 days later the supernatant fluid was harvested. Virions were purified by centrifugation in a discontinuous sucrose gradient as described by Norrby and Hammarskjöld (9).

Radioactivity counting. Twenty-five to 100-µliter samples were mixed in a polyethylene scintillation vial with 10 ml of a scintillator solution (2,5-diphenyloxazole, 7 g; dimethyl-1,4-bis-2-[5-phenyloxazolyl]benzene, 0.6 g; ethylene glycol monoethyl ether, 300 ml; naphthalene, 150 g; toluene, 550 ml), and counted in a liquid scintillation counter.

Determination of protein concentration. The method of Lowry et al. (5) was used with bovine serum albumin (Armour) as a standard.

RESULTS

Adsorption of adenovirus hexon proteins to microplates. A 3H-Ad 3 hexon solution (205 µg of protein/ml; 21,560 counts per min per 25 µliters; hexon antigen complement fixation titer, 1:128) was diluted quadruply in microplates with different diluents. After incubation at room temperature for 1 h, the fluids from the four wells at each dilution were pooled and 50 µliters was taken out for radioactivity counting. The plates were sealed with tape and placed overnight in a refrigerator. Then a 25-µliter sample was taken from the rest of the pool and counted for radioactivity.

A great decrease of the radioactivity from the theoretical value was observed if PBS without proteins was used as a diluent (Fig. 1). The decrease became particularly pronounced with prolonged incubation. However, if proteins were added to the diluent, virtually no decrease was observed. This means that hexon adsorbed to microplates but that adsorption was effectively prevented by the proteins added to the diluent as a carrier.

Adsorption of measles and Ad 2 virions to microplates. The microplates used in the experiments were made of polystyrene, which is hydrophobic in nature. It was considered of interest to determine whether there is a different tendency for adsorption between lipoproteins and ordinary proteins. For this purpose measles virions, which contain lipids, and Ad 2 virions, which do not, were used.

A measles virion suspension (78 µg of protein/ml; 5,640 counts per min per 25 µliters; hemagglutination [HA] titer, 1:32) and an Ad 2 virion suspension (134 µg of protein/ml; 10,470 counts per min per 25 µliters; HA titer, 1:1,600) were diluted 100-fold in polyethylene vials with different diluents. The diluted suspensions were immediately transferred with a Biopette to microplates (25 µliters per each well). After incubation at room temperature for 1 h without agitation, the fluids of six wells were pooled, and 100 µliters was taken for radioactivity counting. The plates were further incubated overnight in a refrigerator. Then the fluids of another six wells were pooled and 100 µliters of the pool was counted for radioactivity.

Both measles and Ad 2 virions adsorbed to the microplates in the absence of carrier proteins in the diluents. However, gelatin, BSA, or CS effectively prevented the adsorption (Fig. 2).

DISCUSSION

Protein adsorbs to the surface of glass and plastics (1, 3). Glass tubes used for the macromethod have a hydrophilic surface with an electrical charge. Conversely, polystyrene, of which microplates are made, has a hydrophobic surface with no electrical charge. Proteins may have different tendencies of adsorption to glass and polystyrene. Gamma globulins adsorb to polystyrene (3), and this phenomenon has been utilized for solid-phase radioimmunoassay (1, 2, 4, 9).

The adsorption phenomenon is of practical importance in titration methods in which end points are determined by dilution. The relative rate of protein adsorption becomes markedly greater at lower concentrations. Thus, when purified proteins with high biological activity are titrated by the "dilution" method, a great reduction of the end-point titers from an expected value might be observed if the diluent does not contain carrier proteins. Conversely, when a mixture of proteins such as serum or unpurified preparations of hemagglutinins (HAs) is used, other proteins in the mixture which have a higher adsorbability might prevent the adsorption of antibodies and HAs.

Since the importance of carrier proteins in the diluent was confirmed, gelatin (0.01%) is being used in our laboratory in the diluents for most HA tests. Gelatin was preferred to BSA since adherence of erythrocytes to the bottom surface of microplates could not be prevented by 0.01% BSA, but could by 0.01% gelatin. However, gelatin is not used in HA tests for Ad 3 since it has some inhibitory effect on HAs of Ad 3 but not Ad 2 (Inouye, unpublished data).

There are other features peculiar to the microtitration method which should be considered when performing the tests. The smaller volume handled in the micromethod more
readily introduces error. Sasson and Kennes (11) pointed out that “rapid mixing” may produce a small bubble in the microdiluters, thus reducing the pick-up volume of the diluter and lowering the titers obtained. In addition, the conditions for HA reactions in the micromethod are not completely the same as in the tube method. Suppose that the reaction volume of 0.2 ml in the macromethod is reduced to 0.025 ml in the micromethod (see Fig. 3). Thus, the macro-versus-micro ratio of volume (V) is 1:18; the ratio of height (h) is 1:1.8; the ratio of surface (S) is 1:4; and the ratio of (S/V) is 1:2. This means that red blood cells settle to the bottom of the microplates in half the time required for the macromethod. If the reaction between HAs and red blood cells is very slow, a portion of red blood cells may reach the bottom of the well before completion of the reaction, thus causing a lower HA titer or an unclear end point with partial hemagglutination patterns in more than one dilution. Ad 2 HA attachment to red blood cells is slow compared with that of Ad 3 HAs. Complete mixing is important for promoting HA attachment (Inouye and Norrby, unpublished data).

Fig. 1. Adsorption of adenovirus hexon proteins to microplates. A purified "H-Ad 3 hexon solution (205 μg of protein/ml; 21,560 counts per min per 25 μlitters) was diluted in polystyrene microplates with different diluents. Pooled samples of each dilution were counted after 1 and 24 h of incubation. ---, Theoretical line of twofold serial dilution; ●—●, PBS without protein; □—□, PBS + 0.01% gelatin; Δ—Δ, PBS + 0.01% BSA; ○—○, PBS + 0.2% (vol/vol) calf serum.
FIG. 2. Adsorption of measles and Ad 2 virions to microplates. A highly diluted suspension of purified $^3$H-measles virion (0.8 µg of protein/ml) and of purified $^3$H-Ad 2 virion (1.3 µg of protein/ml) were put into microplate wells (25 µl per each well). After 1 and 20 h of incubation, pooled samples (0.1 ml each) were counted. The same symbols shown in Fig. 1 were used.

**Macro-versus-Micro Ratio**

![Diagram](image)

The ratio of $V = 0.2 \text{ ml} : 0.025 \text{ ml} = 1:8$

$h = \frac{h_1}{h_2} = \frac{\sqrt{1/6}}{\sqrt{1/8}} = 1:1/2$

$S/V = \frac{(1/2)^2}{1/4} = 1:1/2$

Sometimes it may be advantageous to reshake microplates (13). There is a thinner film of agglutinated cells in hemagglutination-positive wells in the micromethod than in the macromethod. Also, the surface area of the reaction mixture in the micromethod is relatively larger, causing greater adsorption of proteins to microplate wells and a higher evaporation rate.

**ACKNOWLEDGMENTS**

I am grateful to Erling Norrby, Karolinska Institutet, for valuable discussions and criticism, and to Ylva Gollmar for preparing measles virions.

This investigation received financial support from the World Health Organization.

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