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Classification of Hybridomas to Respiratory Syncytial Virus Glycoproteins (41509)

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Abstract. We have classified 28 hybridomas to the RS virus glycoproteins, VP66, VP84, by virus neutralization, RIP, and RIA tests. Without resorting to RIP, a combination of neutralization tests and RIA on BCH4 and RS/HO cells could be used to classify 79% of the hybridomas antibodies correctly. VP66 contains major determinants for virus neutralization since 11/13 hybridoma antibodies to this protein neutralized RS virus while 0/15 hybridoma antibodies to VP84 neutralized the virus.

Respiratory syncytial (RS) virus is an important respiratory pathogen of the family Paramyxoviridae (1) and is a leading cause of lower respiratory disease in young children (2). Before a successful RS virus vaccine can be developed, more information about the surface proteins is needed in order to fully define the host response to RS virus infection. Such studies have been hampered by the difficulty in obtaining purified viral proteins and monospecific antisera. We have investigated these problems by developing hybridomas to glutaraldehyde-fixed RS virus-infected HeLa cells. The initial fusion resulted in seven clones secreting monoclonal antibodies to three viral proteins (3).

The development of Balb/c cells persistently infected with RS virus (BCH4) (4) provided a convenient substrate for radioimmunoassay (RIA) and allowed us to eliminate clones secreting antibodies to human components early in the screening process. These cells potentially could induce antibodies better if injected into a syngeneic host to minimize antibody response to cellular antigen. We, therefore, injected Balb/c mice with syngeneic BCH4 cells for the production of viral antigen-specific hybridomas.

Although there has been a number of studies involved with the identification of RS virus glycoproteins (5–11) the results are not straightforward and are often contradictory. Most authors present data for three glycoproteins with molecular weights of 70,000–90,000, 40,000–50,000, and 19,000–25,000. Wunner and Pringle (11), however, could not confirm the presence of the 70,000–90,000 or 19,000–25,000 mol wt glycoproteins, but instead found two glycoproteins, mol wt 40,000–50,000 (VGP48 and VGP42). Recently the large glycoprotein was immunoprecipitated from infected cells (5, 6) but other glycoproteins, mol wt 45,000–50,000 and 17,000–24,000, were not. The development of monoclonal antibodies with specificity for the RS virus glycoproteins would greatly help in the identification and study of these glycoproteins.

Materials and Methods. *Cells and viruses.* HeLa Ohio (HO) cells were obtained from Flow Laboratories (McLean, Va.). The Balb/c cell line (BCH4), persistently infected with RS virus, has been described (4). Both cell lines were grown in equal parts of minimum essential medium with Earles salts and basal Eagle medium with Hank's salts, 10% heated (56°/45 min) fetal bovine serum (Dutchland Labs) and 4 m*M* glutamine. Suspension cultures of HO cells were grown in 10% heated fetal bovine serum, 4 m*M* glutamine in Eagles minimum essential medium, spinner modified. HO cells were infected (RS/HO) in suspension with 1 PFU/cell of RS virus, Long strain. The cells were rinsed 2 hr after infection and resuspended in suspension medium, with 5% fetal bovine serum, at 2×10^6

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cells/ml. RS/HO cells were labeled with 10 μCi/ml of D-[1,6-^{3}H]glucosamine hydrochloride (New England Nuclear, sterile aqueous solution) 12 hr postinfection. Cells and culture fluid were collected 24 hr postinfection. Virus-free culture fluid was prepared by pelleting the virus at 7500 rpm for 4 hr in a Beckman J-21C centrifuge (12).

Virus neutralization. Hybridoma culture fluids were tested for virus neutralizing antibodies as described (3) with minor modifications. Four units of guinea pig complement (Flow Laboratories) were added throughout to increase the sensitivity of the assay (13).

Indirect immunofluorescence (IFL). IFL was performed using live RS/HO cells as described (4).

Radioimmunoprecipitation (RIP). RIP using iodinated viral proteins (14), [^{3}H]-glucosamine-labeled cell lysate, or virus-free culture fluid from [^{3}H]glucosamine-labeled RS/HO cells was performed as described (14). Samples were analyzed by slab gel electrophoresis (14).

Radioimmunoassay (RIA). Indirect RIA using ^{125}I-goat anti-mouse γ globulin as the second antibody and methanol-fixed RS/HO or BCH4 cells as the substrate was recently described (3).

Somatic cell hybridizations. The generation of hybridomas followed standard techniques as described for RS virus, except viable BCH4 cells were used as the immunogen instead of glutaraldehyde-fixed RS/HO cells (3).

Results. Approximately 1000 hybridoma colonies were originally screened by RIA using methanol-fixed BCH4 cells. Ten percent appeared to be RS virus specific; these colonies were cloned and virus neutralization, RIP, IFL, and further RIA analysis using methanol-fixed BCH4 and RS/HO cells performed on the spent culture fluid.

Immunofluorescence. All of the 28 clones chosen for further study had the staining characteristics of surface proteins as determined on live RS/HO cells (4). Typical results are shown in Fig. 1.

Radioimmunoprecipitation. RIP analysis indicated that about 75% of the clones precipitated one of two viral glycoproteins (Fig. 2, Table I); designated VP84 and VP66

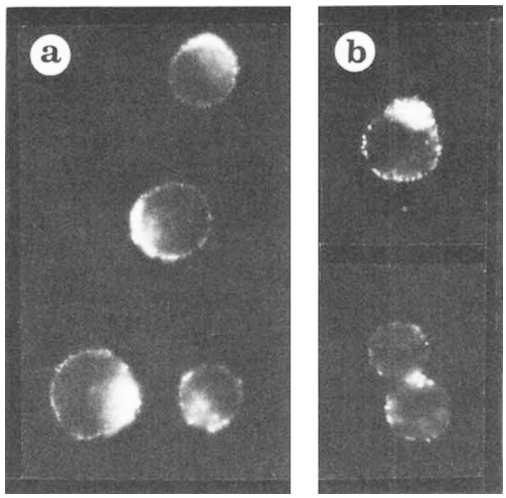

FIG. 1. Living cell immunofluorescence of RS/HO cells. RS/HO cells were prepared for living cell immunofluorescence as described (4) 22 hr after infection. The cells were incubated with (A) monoclonal 111, class 84A; (B) monoclonal 13-1 class 66A.

(14). VP84, a protein not present in great abundance on the virion (7, 14) has been identified using RIP (14). VP66 consists of two disulfide-bonded polypeptides with molecular weights of 43,000 and 19,000 (14); both polypeptides are glycosylated, VP19 relatively more so than VP43. Seventy-seven percent of the clones specific for VP66 could precipitate iodinated viral VP66 protein while only 1 of 15 (6%) clones specific for VP84 could precipitate iodinated VP84 (Table I). Seventy-three percent of the clones specific for VP84, however, precipitated [^{3}H]glucosamine-labeled VP84 (Table I). RIP using [^{3}H]-glucosamine-labeled RS/HO cell lysates showed the presence of VP66 (VP43 and VP19) when precipitated by monoclonal antibodies specific for VP66. However, VP84 and an additional glycoprotein with a mol wt of 43,000 to 46,000 precipitated from cell lysates by monoclonal antibodies specific for VP84. This protein, which is not readily detected in virus-free culture fluid, may represent a cleavage product of VP84 (Fig. 2). The width and very diffuse nature of the protein band is very similar to that obtained with VP84, but markedly different from VP43, indicating that VP43 has not coprecipitated with

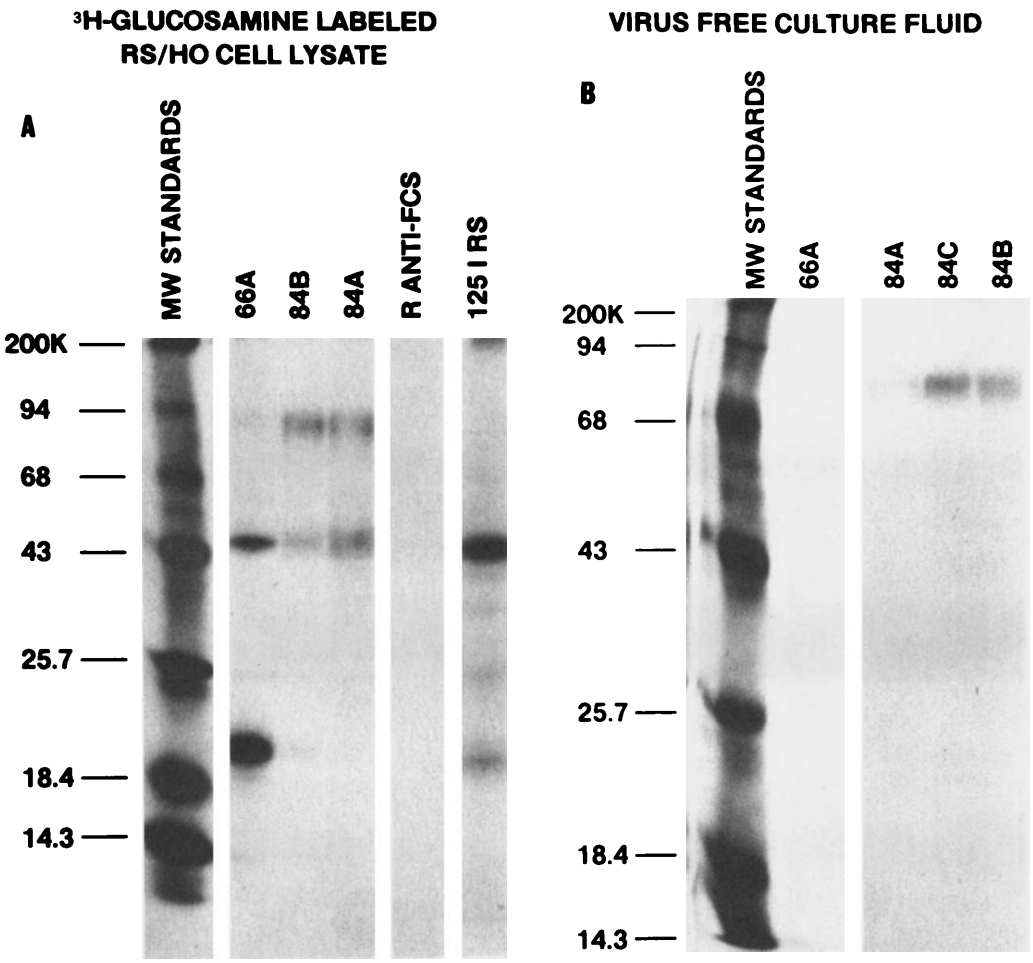

FIG. 2. RIP of RS virus-specific glycoproteins from cell lysates or culture fluid. (A) RS/HO cells grown in suspension were labeled from 12 to 24 hr postinfection with [^{3}H]glucosamine. The cells were pelleted, rinsed twice in Hank's balanced salts, 50 m*M* Hepes, pH 7.5, PMSF 100 mg/ml, and suspended in the same buffer at 5×10^7 cells/ml. Cell lysates were prepared by adding Triton X-100 to 0.1% and incubating at 37° for 30 min. Nuclei and large membrane fragments were removed and the supernatant made up to 0.1% SDS. This mixture was incubated at 45° for 30 min. Approximately 5×10^7 cell equivalents of this cell lysate were incubated with 100 μl of washed Immunoprecipitin (Bethesda Research Labs.), cleared, and incubated with the indicated antibody. The exact RIP procedure has been described (14). Samples for electrophoresis were run on slab gels consisting of 14% acrylamide with DATD as the crosslinker (14), using the discontinuous system of Laemmli (15). The specific antibodies used were as follows: class 66A, RS 13-1; class 84B, RS 18-1; class 84A, RS 111; R anti-FCS, rabbit anti-fetal calf serum (14); ^{125}I RS, iodinated RS virus proteins precipitated with rabbit anti-RS virus (14). ^{125}I-VP84 is present in immunoprecipitates with rabbit anti-RS virus (14) but is not visible in this photograph.

(B) A virus-free culture fluid preparation containing only VP84 was used to screen for monoclonals to this protein. RIP and gel electrophoresis were as described (14). The specific antibodies used were as follows: class 66A, RS 13-1; class 84A, RS 111; class 84C, RS 322; class 84B, RS 821.

VP84. Although Staphylacoccal protease treatment of culture fluid containing VP84 generates a fragment with a molecular weight of 49,000 (S. Spring, personal communication), we have not eliminated the possibility that the coprecipitating protein is due to cellular contamination. If the 40,000–45,000 mol wt protein precipitated

TABLE I. CLASSIFICATION OF HYBRIDOMAS TO RS VIRUS SURFACE PROTEINS

Class	Neut.[a]	RIP[b] V	RIP[b] S	Relative binding ratio[c]	No. of clones
66A	+	+	−	<1	6
66B	+	+	−	1–2	3
66C	+	−	−	~1	2
66D	−	+	−	<1	1
66E	−	−	−	<1	1
84A	−	+	+	2	1
84B	−	−	+	<2.5	4
84C	−	−	+	3–5	6
84D	−	−	−	2	4

[a] Viral neutralization activity scored positive if at least 50% inhibition is found at 1:20 dilution.

[b] RIP performed using: iodinated viral proteins (V), [^{3}H]glucosamine-labeled, virus-free culture fluid from RS/HO cells (S). This fluid was shown to be free of [^{3}H]glucosamine-labeled VP66 by RIP/SDS–PAGE. Viral protein precipitated is indicated by class number. See Fig. 2.

[c] Relative binding ratio is determined from comparative RIA on RS/HO and BCH4 cells per Fig. 3.

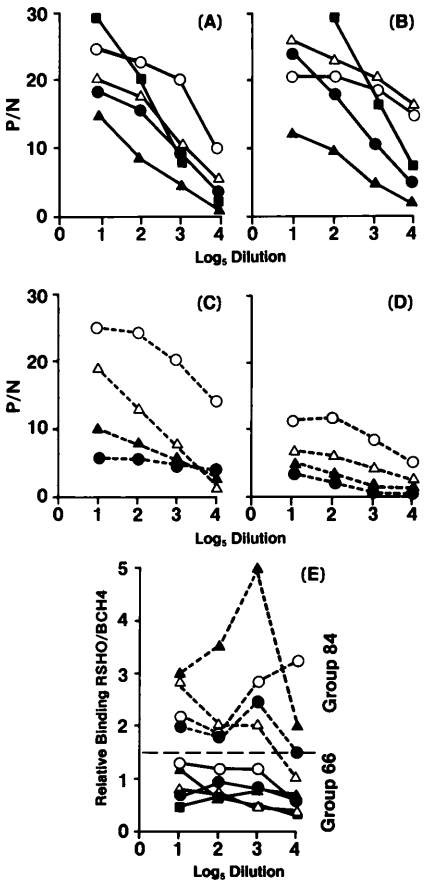

FIG. 3. Characterization of monoclonal antibodies to RS virus glycoproteins by RIA. Assay of antibodies with anti-VP66 activity (——) on RS/HO (A) and BCH4 (B) cells; monoclonal antibody and class identifications in (A) and (B) are (●) RS 284:66A, (○) RS 334:66B, (▲) RS 162:66C, (△) RS 47:66D, and (■) RS 184:66E. Assay of antibodies with anti-VP84 activity (– – –) on RS/HO (C) and BCH4 (D) cells; monoclonal antibody and class identifications in (C) and (D) are (●) RS 111:84A, (○) RS 93:84B, (▲) RS 104:84C, and (△) RS 402:84D. P/N is the cpm for the culture media samples to that for blank culture media (100–175 cpm). Antibodies assayed on uninfected HeLa or Balb/c cells exhibited P/N values ≤2. (E) The relative binding ratio was determined by dividing the P/N number obtained on RS/HO cells by the P/N number obtained on BCH4 cells.

by anti-VP84 antibodies is a cleavage product, then it probably represents the VGP48 of Wunner and Pringle (11) while VP43 is their VPG42. This interpretation is based on the relative level of [^{3}H]glucosamine incorporation into VP43 and VP19 compared to the relative level of [^{3}H]glucosamine into proteins with mol wt of 40,000–50,000 and 19,000–24,000, respectively (5–8, 11).

Virus neutralization. All of the selected clones have been tested for virus neutralization activity (Table I). None of the monoclonal antibodies with neutralizing activity appears to be directed against VP84. Lack of neutralizing activity was not caused by low antibody concentration as indicated by the level of antibody binding in the RIA tests as shown below.

Radioimmunoassay. When clones were compared by RIA using BCH4 or RS/HO cells as substrate consistent patterns in the P/N binding ratios were observed (Fig. 3). These differences were used to classify clones as VP84-like or VP66-like (3). This has been useful but not entirely accurate since there is some overlap between monoclonal groups specific for the two surface proteins. In addition, monoclonals specific for RNP exhibit binding ratios similar to those for VP66 (data not shown). Further testing will be required to eliminate these problems.

Discussion. A fusion using spleenocytes from mice immunized with live BCH4 cells yielded approximately 15 stable clones secreting monoclonal antibodies to each of two surface proteins. We developed a classification scheme for these hybridomas based on IFL, virus neutralization, RIP and RIA. This classification scheme divides monoclonals to VP84 into four subgroups and those to VP66 into five subgroups. In most cases the assignment to a particular subgroup was straightforward. However, a small portion of the clones that failed to specifically precipitate a viral protein have been assigned to a subgroup based on RIA data alone and these assignments must be regarded as tentative. As knowledge about the importance of each subgroup grows, it should be possible to rapidly screen new fusions for the desired hybridomas.

In the interest of developing a rapid, simple screening technique for hybridomas to RS virus surface proteins, a variety of tests have been performed on the 28 hybridomas isolated to date. If initial screening is done using BCH4 and RS/HO cells and this is followed by virus neutralization tests, 79% of the selected clones can be placed in their correct classification. This procedure eliminates the need to screen each initial clone by the cumbersome procedures of IFL and RIP.

We are currently studying reasons for the differential binding of some classes of monoclonal antibodies to BCH4 and RS/HO cells. This may simply reflect differences in the amount of VP84/VP66 in these cells or differences in the orientation of these proteins on the cell surface.

The efficacy of using BCH4 cells as immunogen and substrate for the production and screening of hybridomas to RS virus has been established. The success obtained using BCH4 cells as an immunogen probably stems from the lack of response to BCH4 cellular antigens. In addition, the persistently infected BCH4 cells are providing a continual source of viral antigens for purification and characterization.

VP66 contains major determinant(s) involved in virus infectivity, VP84 apparently does not. We have not been able to assign a definite function to VP84, although this protein has the physicochemical characteristics of the paramyxovirus hemagglutinin (14).

The use of monoclonal antibodies with specificity for VP84 and VP66 should help clarify the complex picture of RS virus glycoproteins. The fact that two antigenically distinct proteins have very similar molecular weight raises some question as to the identity of the 45,000–50,000 mol wt glycoprotein found in nonimmunoprecipitated RS virus or infected cells (7–11). In fact, the protein that coprecipitates with VP84 may represent VGP48 as defined by Wunner and Pringle (11) while VP43 may represent their VGP42. Earlier results obtained by direct analysis of viral proteins has not yet been consolidated with more recent work involving the RIP of viral proteins (5, 6, 14). This is caused, in part, by the variable precipitation efficiency of RS virus proteins (5, 14) resulting in a selection of viral proteins that may or may not be present in great abundance. The selection process, coupled with the presence of viral proteins having similar molecular weight (e.g., VGP48, VGP42; 11), VP43, postulated cleavage product of VP84 (this paper) has produced considerable uncertainty about the number and size of the RS virus glycoproteins (5–11). This uncertainty has prevented a comprehensive understanding of RS virus glycoproteins from being developed. Hopefully, this situation will be clarified as monoclonal antibodies are used to examine the glycoproteins of RS virus more extensively.

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