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Effect of Anticellular Serum on the Attachment of Enteroviruses to HeLa Cells[1]

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Anticellular serum (ACS), in the absence of an active complement system, was shown to inhibit the attachment of poliovirus types 1 and 2, echovirus type 6, and coxsackievirus types A13, B1, and B3 to viral receptors of live HeLa cells. This is the first report to provide evidence that ACS has an inhibitory effect on the interaction between host cells and coxsackieviruses of group B. The titer of inhibitory activity of ACS varied inversely with the cell concentration used, the reaction being virtually completed after an incubation period of 30 min at 37 C. The inhibitory activity of ACS persisted for more than 4 hr at 37 C, and was shown to be reversible at *p*H 2.0, revealing that although the receptors for attaching virus were inactivated by ACS the inactivation was not permanent. These findings are consistent with the concept that antibodies in the ACS combine with and blockade viral receptors located at the cell surface. An antiserum with a specificity for inhibiting attachment of coxsackievirus B1 was obtained by dual absorption of ACS with cells saturated with coxsackievirus type B3 and chymotrypsin-treated cells. These findings offer an approach whereby the antigenic relationship of viral receptors to other constituents of the cell surface can be studied.

Bacterial cells treated with an antibacterial serum have been shown to become resistant to the action of bacteriophages and colicins (2, 3, 10). Similarly, anticellular serum (ACS) has been shown to inhibit infection of animal cells in culture by a variety of viruses (1, 4, 11, 22, 25), including enteroviruses (12, 15, 23, 26, 27, 28). The results of studies with enteroviruses, however, have been somewhat inconsistent; Habel et al. (12), Quersin-Thiry (23), and Holland and McLaren (15) all showed a significant reduction in poliovirus infectivity for cells treated with ACS, whereas Timbury (26) found only a minimal inhibition. In addition we found it interesting that these studies revealed no inhibitory effect or only a minimal inhibitory effect of ACS to infection by coxsackieviruses of group B, under conditions similar to those in which echoviruses and coxsackieviruses of group A were greatly affected. Although the inhibitory effects of ACS on enterovirus infection were attributed primarily to inhibition of virus attachment to susceptible cells, little direct experimental evidence has been presented to support this hypothesis. It was for these reasons, and the thought that assorted enterovirus receptors of living HeLa cells could be differentiated further by an antigenic analysis (7-9, 24, 30, 31), that the following study was initiated.

MATERIALS AND METHODS

Viruses and virus assay. The origin of strains of coxsackievirus types B1, B3, and A13 and of poliovirus types 1 and 2 used in this investigation and the method of assay have been described previously (7, 9). Echovirus type 6 was obtained from Morton Klein, Temple University School of Medicine, Philadelphia, Pa.

Cell lines. Sublines of HeLa cells designated HeLa (MBA) and HeLa (JJH) were grown as monolayer cultures in medium containing calf serum, as described by Zajac and Crowell (30). Although both cell lines were comparable in their susceptibility to the viruses used, except for coxsackievirus type A13, the experiments were performed with HeLa (MBA) cells. Virus assays, except for A13 virus, were performed routinely with monolayers of HeLa (JJH) cells, since these cells gave more uniform monolayers and remained firmly attached to the glass following manipulations required

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for the plaque assay. An additional cell line designated ML (5) was received from D. A. Buthala of the Upjohn Co., Kalamazoo, Mich.; this cell line was used only for the plaque assay of coxsackievirus type A13, since these cells retained a relatively high and uniform susceptibility to plaque formation by A13 virus.

Determination of cell concentration. Cells grown in monolayers were rinsed twice with phosphate-buffered saline (PBS), free from magnesium and calcium, and were suspended by use of 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS, *p*H 7.8, and a rubber scraper. The cells were deposited by centrifugation, resuspended in an appropriate volume of Hanks balanced salt solution (BSS), and enumerated with the Coulter counter model B.

Determination of receptor activity of HeLa cells. HeLa cells in suspension, usually at a final concentration of either 5×10^6 or 10^7 per ml, were incubated with virus to give an input multiplicity of infection of 0.1 or less. After incubation at 37 C for 1 hr, a sample of the virus-cell mixture was diluted 100-fold, the cells were removed by centrifugation, and the amount of virus attached to the cells was calculated by the difference between the amount of free virus found and the input virus (8). To measure initial virus content, a control tube containing the virus inoculum and BSS with 3% calf serum, in place of the cell suspension, was included in each experiment. No soluble receptor activity was detected in the fluid phase of the system.

Enzymatic treatment of cells. HeLa cells were treated with freshly prepared solutions of trypsin and α-chymotrypsin in order to inactivate the cellular receptor activity for poliovirus and coxsackieviruses of group B, respectively, according to the method of Zajac and Crowell (30), with the exception that, after enzyme treatment, the cells were diluted and washed in BSS containing no calf serum.

Preparation of (ACS). Normal HeLa cells and HeLa cells treated with either trypsin or chymotrypsin were used as antigen to immunize rabbits. Samples of serum were collected from each animal prior to immunization. Each rabbit was injected intramuscularly once a week with approximately 2×10^8 cells, suspended in 2.0 ml of BSS and emulsified in an equal volume of Freund's complete adjuvant (Difco), for a period of 6 successive weeks. One week after the last injection and at two subsequent weekly intervals, the rabbits were bled from the central ear artery. The serum samples collected from each rabbit were pooled separately and were stored at −24 C until used.

Assay of anticellular serum by inhibition of virus attachment. HeLa cell monolayers grown in 32-oz prescription bottles were washed twice with PBS and were suspended by use of EDTA and a rubber scraper. The cells were sedimented by centrifugation at $350 \times g$ for 10 min, resuspended in BSS, and counted. Samples of 10^7 cells were distributed to separate tubes and sedimented by centrifugation; the cells were then resuspended in 5.0 ml of ACS diluted in BSS and incubated at 37 C for 1 hr. The treated cells were washed twice with BSS, centrifuged, and resuspended in 0.9 ml of BSS containing 3% calf serum to receive 0.1 ml of virus [10^6 plaque-forming units (PFU)]. The amount of virus which attached within 1 hr was determined as described previously. The reciprocal of the ACS dilution which inhibited the amount of virus attachment by 50 (± 5) %, as compared to the amount of virus attached to untreated cells, was designated as the ACS titer. All antisera used in these experiments were heated at 56 C for 30 min before being used, to inactivate complement, except where indicated, and were shown to be free from virus-neutralizing activity.

In some experiments, the inhibitory activity of ACS was assayed in a manner similar to that above, except that cells in monolayer cultures were used. HeLa cells growing in monolayers in 2-oz French-square bottles were drained of growth medium, overlaid with dilutions of ACS, and incubated for 60 min at 37 C. The excess ACS was decanted, and the monolayers were washed two times with PBS and drained thoroughly. We added 0.2 ml of coxsackievirus B3, containing 2×10^5 PFU to each monolayer. The monolayers were incubated at 37 C for 60 min; then 19.8 ml of BSS containing 3% calf serum was added to each culture to stop attachment, and the fluids were decanted and frozen at −24 C until assayed for unattached virus.

Absorption of anticellular serum with cell antigen preparations. Normal HeLa cells and HeLa cells treated either with proteolytic enzymes, to selectively inactivate virus receptors, or with saturating levels of virus, to blockade specific virus receptors, were used as antigens to absorb ACS. For preparation of enzyme-treated cell antigens, HeLa cells were treated with either chymotrypsin or trypsin, as described previously, and the cells were shown to be devoid of receptor activity for coxsackievirus type B3 and poliovirus type 1, respectively. The methods described by Crowell (8) were used to prepare HeLa cells saturated with coxsackievirus type B3 and to measure the extent of receptor blockade by the virus attachment-interference assay. For absorption, ACS was diluted 1:5 or 1:50 in BSS and was added to an equal volume of a cell preparation suspended in BSS to give a final cell concentration of 2×10^7 cells per ml. The ACS-cell mixture was prepared in a 16-oz prescription bottle, and was incubated for 1 hr at 25 C in a horizontal position and for an additional 18 hr at 4 C on a mechanical shaker. The mixture was transferred to a tube, the cells were sedimented by centrifugation ($900 \times g$), and the absorbed antiserum was withdrawn and stored at −24 C until used. Where indicated, ACS was absorbed first with enzyme-treated cells and then with cells saturated with coxsackievirus type B3. Because it was found that cellular receptors for B1 virus remained blocked by B3 virus for long periods of time at low temperatures, ACS was absorbed as described above, except that the cell-serum mixture was incubated at 4 C for 24 hr. The amount of infectious B3 virus found in the absorbed ACS (from 4×10^6 to 7×10^6 PFU per ml) was not sufficient to account for the specific inhibitory activity associated with the ACS (8), and was either inactivated by ultraviolet irradiation prior to assay of homologous challenge virus, or was neutralized by homotypic antiviral serum prior to assay of heterologous virus.

RESULTS

Effect of anticellular serum on the attachment of enteroviruses to HeLa cells. Preliminary experiments were performed to determine the ability of ACS to inhibit the attachment of coxsackievirus type B3 and poliovirus type 1 to HeLa cells. HeLa cells at a final concentration of 5×10^6 per ml were suspended and treated for 1 hr at 37 C with a 1:30 dilution of ACS. Cells that were treated with either preimmunization serum diluted 1:10 or with BSS served as controls. After incubation, the ability of treated and control cells to attach poliovirus type 1 and coxsackievirus type B3 was determined. The results of twelve separate experiments showed consistently that the 1:30 dilution of ACS inhibited the attachment of both enteroviruses tested by greater than 95%, whereas cells treated with preimmunization serum or BSS attached virus normally.

A 1:30 dilution of ACS was not cytotoxic in the absence of added complement. As a point of reference of the potency of the ACS prepared in the present study, the cytotoxic titer was determined by the method of Hirata (14). Assays in which 2×10^6 cells per ml were treated with increasing dilutions of ACS in the presence of guinea pig complement for 1 hr at 37 C showed repeatedly that a 1:800 dilution of ACS reduced the number of HeLa cells by 50%.

To determine the optimal conditions for assay of the inhibitory activity of ACS for viral receptors of HeLa cells, each of the experimental conditions, cell concentration and time and temperature of incubation, was examined. Before this could be done, however, it was first necessary to establish the value of selecting a 50% end point for assaying the inhibitory activity of ACS. Diminishing concentrations of ACS in BSS were used to treat HeLa cells at a final concentration of 5×10^6 cells per ml for 1 hr at 37 C. The cells were washed free from unreacted serum components by centrifugation in BSS and were tested for the amount of coxsackievirus type B3 with which they could bind in 1 hr at 37 C, when used at a final concentration of 5×10^6 cells per ml. The results of three separate experiments, which are averaged and presented in Fig. 1, show that the amount of inhibition of virus attachment was dependent upon the amount of ACS used to treat the cells, and that a 50% inhibition of receptor activity would provide a sensitive and reproducible point for titration of ACS activity. The results of a representative experiment showing the influence of cell concentration on the titer of ACS are presented in Fig. 2. It can be seen that, over the range of cell concentrations used, a twofold

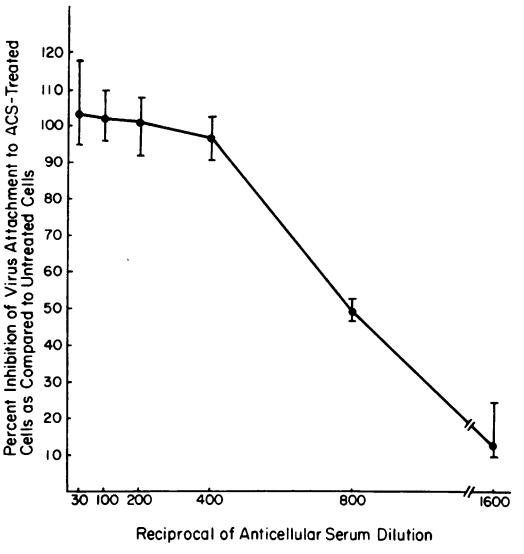

FIG. 1. *Relationship between the concentration of anticellular serum and the amount of inhibition of attachment of coxsackievirus type B3 to HeLa cells.*

increase in cells resulted in a proportionate decrease in ACS titer. Similar results were obtained on three separate occasions. Although the use of 10^6 cells per ml resulted in the highest titer of ACS, a concentration of 2×10^6 cells per ml was employed in further experiments in order to conserve the amount of ACS used and to obtain a more efficient recovery of cells after treatment. Experiments designed to determine the effect of different incubation times, from 5 to 120 min, on the extent of interaction between ACS and cells revealed that the titer of ACS was maximal after an incubation period of 30 min, and that prolonged incubation resulted in no additional increase. To ensure a maximal effect of ACS and to obtain uniformity of experimental conditions, an incubation period of 1 hr was selected as standard. An incubation temperature of 37 C for 1 hr was found to result in a twofold higher titer than that obtained for a comparable period at 4 C. Therefore, 37 C was used as the standard incubation temperature.

To determine the capacity of ACS to inhibit attachment of additional enteroviruses (coxsackievirus types B1, B3, A13; poliovirus types 1, 2; echovirus type 6) to HeLa cells, experiments were performed under the conditions found to be optimal for inhibition of coxsackievirus B3. The data presented in Table 1 show that ACS effectively inhibited the attachment of all of the enteroviruses tested to HeLa cells.

Effect of anticellular serum on production of

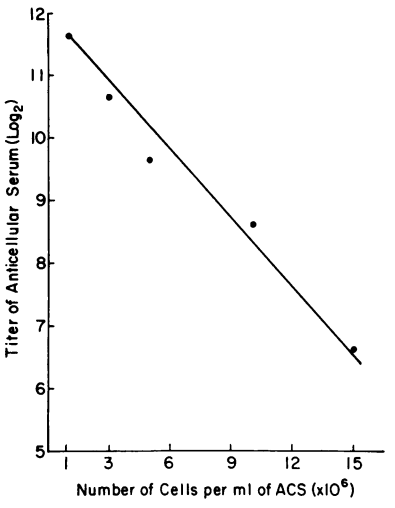

FIG. 2. *Effect of cell concentration on the titer of anticellular serum for inhibiting attachment of coxsackievirus type B3 to HeLa cells in suspension.*

plaques by enteroviruses. Next, it became important to resolve the apparent discrepancy between the above findings, which showed ACS capable of inhibiting virus attachment to cells, and those of Timbury (26), who showed ACS to have no effect or only a minimal effect on plaque production by the three types of polioviruses and the coxsackieviruses of group B. Experiments were performed to test the ability of ACS to inhibit plaque production by coxsackievirus type B3 and poliovirus type 1. The procedure of Timbury (26) was followed closely, with the exception that HeLa cell monolayers and homotypic ACS were used in place of the human amnion cell system.

Dilutions of ACS were prepared in BSS, and 0.3-ml amounts were added to each of a series of freshly washed monolayers containing 8×10^6 HeLa cells growing in 2-oz bottles. Growth medium, instead of ACS, was used as the control. After exposure to ACS for 1 hr at 37 C, the excess fluid was removed, and 0.1 ml of virus containing approximately 100 PFU was added to each bottle. For virus attachment, a period of 1 hr at room temperature was used; then the monolayers were covered with 5 ml of agar-containing medium and were incubated at 37 C for development of plaques. The results given in Table 2 show that the degree of inhibition of plaque production obtained by pretreatment of HeLa cell monolayers with ACS was minimal, but comparable to that reported by Timbury (26). An additional experiment was performed to test the possibility that cells in monolayer were influenced by ACS in a manner different from cells in suspension.

TABLE 1. *Inhibition of attachment of assorted enteroviruses to HeLa cells by anticellular serum*

Virus	Titer of ACS[a]
Coxsackievirus	
B1	1,200
B3	1,600
A13	400
Poliovirus	
T1	200
T2	400
Echovirus	
T6	3,200

[a] The reciprocal of the dilution of anticellular serum that inhibited the attachment of virus by 50% under standard conditions.

HeLa cells grown in monolayers in 2-oz bottles were treated with dilutions of ACS as before, except that volumes of ACS ranging from 0.3 ml to 4.0 ml were used to achieve different cell concentrations relative to the fluid volume, and the monolayers were tested for the capacity to attach coxsackievirus type B3, as described in Materials and Methods. The results of this experiment (Table 3) show that the titer of ACS for inhibiting virus attachment to cells in monolayers was influenced by cell numbers as before, with the ACS titer being approximately fourfold lower than when tested on cells in suspension. A further comparison of the relationship between the capacity of ACS to inhibit attachment of virus and to inhibit plaque formation is presented in the Discussion.

Persistence of viral-attachment inhibition produced by ACS. It has been demonstrated that cells treated with ACS lose their sensitization to lysis unless complement is introduced into the system within 1 hr of addition of the antiserum (19). Experiments were performed to determine the length of time during which ACS would suppress attachment of coxsackievirus type B3 to HeLa cells in suspension.

HeLa cells were suspended in a 1:800 dilution of ACS to give a final concentration of 2×10^6 cells per ml and were incubated at 37 C for 1 hr. The cells were washed by centrifugation in BSS and were tested for the ability to attach coxsackievirus type B3 at 37 C over a 4-hr period. Cells treated either with preimmunization serum or with growth medium containing 3% calf serum, instead of ACS, served as controls. Results similar to those presented in Fig. 3 were obtained on two separate occasions. The inhibition of virus attachment produced by ACS persisted for the entire 4-hr period. An additional experiment of similar design revealed that cells treated with ACS re-

TABLE 2. *Effect of anticellular serum on plaque production by poliovirus type 1 and coxsackievirus type B3*

Virus	Reciprocal of ACS dilution	No. of plaques formed	Avg no. of plaques	Plaque reduction[a]
				%
Poliovirus T1	30	51, 65, 51	56	27
	60	89, 77, 75	80	0
	120	78, 86, 73	79	0
	GM[b]	66, 89, 73	76	0
Coxsackievirus B3	30	75, 69, 76	73	27
	60	96, 93, 82	90	10
	120	110, 105, 98	104	0
	GM[b]	116, 95, 90	100	0

[a] The per cent reduction by ACS in the number of plaques formed was calculated by the expression: 100 − (avg no. of plaques by ACS-treated cells/avg no. of plaques by GM-treated cells × 100).
[b] Growth medium.

mained refractory to virus attachment after incubation for 24 hr at 4 C.

Reversal by acid pH of virus-attachment inhibition produced by ACS. Since enterovirus receptors have been shown to be limited to the surface of HeLa cells (31), it was considered likely that antibodies in the ACS used in the present studies combined with viral receptors of cells to cause the observed inhibition of virus attachment. Experiments were done in order to determine whether the inhibition of receptor activity by ACS could be reversed by use of a low *p*H environment, since it has been shown that antigen-antibody complexes can be dissociated under these conditions (13, 18), and that the HeLa cell receptors for coxsackievirus type B3 are stable at *p*H levels as low as *p*H 1 (Zajac and Crowell, Bacteriol. Proc., p. 133, 1966).

HeLa cells were suspended in 2.0 ml of ACS diluted 1:200 in BSS at a final concentration of 5×10^6 cells per ml, and were incubated for 1 hr at 37 C. The ACS-treated cells were washed twice with BSS, collected by centrifugation, resuspended in a 10-ml volume of 0.05 M glycine buffer (*p*H 2.0), and immediately sedimented again by centrifugation. Finally, the cells were resuspended at a concentration of 5×10^6 cells per ml in BSS, containing sufficient sodium hydroxide to adjust the final *p*H to 7.4, and were tested for the ability to attach coxsackievirus type B3. Additional samples of cells, which were tested in parallel and treated separately with ACS, glycine buffer, or BSS, served as controls. The amounts of virus which attached to the respective cell samples in 1 hr at 37 C are given in Table 4. Similar results were obtained on two separate occasions. The data revealed that a brief exposure of ACS-treated cells to *p*H 2.0 completely reversed the inhibition of virus receptor activity.

TABLE 3. *Effect of cell concentration on the titer of anticellular serum for inhibiting attachment of coxsackievirus type B3 to HeLa cells in monolayer cultures*

No. of cells per ml of ACS	Titer of ACS[a]
1.2×10^6	960
2.5×10^6	480
5.0×10^6	240
17.0×10^6	120

[a] The reciprocal of the dilution of anticellular serum that inhibited the attachment of virus by 50% under the experimental conditions.

Immunological and serological activity of cell antigen preparations. The finding that the proteolytic enzymes trypsin and chymotrypsin could differentially inactivate the receptors on live HeLa cells for poliovirus type 1 and coxsackievirus type B3, respectively (30), made possible an attempt to prepare anticellular serum with the corresponding receptor specificity. Large numbers of HeLa cells were treated with trypsin or chymotrypsin, as described previously, and were used separately as antigen to immunize rabbits. The resulting preparations of anticellular serum were tested for the ability to inhibit the attachment to HeLa cells of poliovirus type 1, coxsackievirus type B3, and echovirus type 6. The results of these experiments (Table 5) revealed that antiserum derived from rabbits immunized with chymotrypsin-treated cells contained only a very low antibody content directed toward the coxsackievirus type B3 and echovirus type 6 receptors, while possessing a reduced, though significant, reactivity for poliovirus receptors. These results were anticipated, since chymotrypsin totally inactivates the B3 receptors, while causing some cross-inactivation

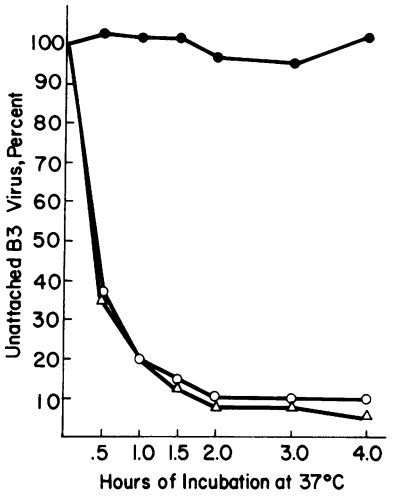

FIG. 3. *Persistence of anticellular serum at the cell surface, as evidenced by inhibition of attachment of coxsackievirus B3. Cells were suspended in anticellular serum (●), preimmunization serum (○), or growth medium containing 3% calf serum (△).*

TABLE 4. *Acid reversal of the inhibition of coxsackievirus B3 attachment by anticellular serum*

Treatment of cell sample	Amt of virus attached
	%
ACS	<5
ACS → *p*H 2[a]	78
*p*H 2	78
BSS	92

[a] Cells treated with ACS, followed by exposure to glycine buffer, *p*H 2.

of poliovirus receptors (30). Also, it was not surprising that the antigenicity of the echovirus type 6 receptor was destroyed by chymotrypsin, since echovirus receptors of HeLa cell debris have been found to be inactivated by chymotrypsin (20). The low titer of the ACS prepared to chymotrypsin-treated cells did not result from an inability of the rabbit to make antibody, since the same animal, after two booster immunizations with normal HeLa cells, gave an ACS titer of 400 against both coxsackievirus type B3 and echovirus type 6. On the other hand, the ACS produced in response to immunization with trypsin-treated cells gave very unexpected results. This antiserum was not only able to inhibit attachment of coxsackievirus type B3, but it also inhibited attachment of poliovirus type 1 to a titer equivalent to that obtained for ACS prepared against normal HeLa cells.

In addition to using the enzyme-treated cells as immunizing antigens, we also tested them for the ability to absorb specific antibodies from ACS

TABLE 5. *Production of antibodies to enterovirus receptors by use of enzyme-treated HeLa cells as antigen*

Treatment of cells for immunization	Titer of ACS found to inhibit attachment of[a]		
	Poliovirus T1	Coxsackievirus B3	Echovirus T6
Chymotrypsin...	50	10	<10[b]
Trypsin.....	200	400	ND[c]
Untreated.....	200	1,600	3,200

[a] The reciprocal of the dilution of ACS that inhibited the attachment of virus by 50% under standard conditions.
[b] The lowest serum dilution tested.
[c] Not done.

prepared against normal HeLa cells. The results of these experiments (Table 6) are consistent with the findings of the preceding experiments; chymotrypsin-treated cells did not absorb antibody directed to the coxsackievirus type B3 receptors, but reduced by greater than twofold the antibody level to the poliovirus receptors. Trypsin-treated cells reduced the antibody levels to both T1 and B3 receptors. It is apparent that the differential use of enzyme inactivation to prepare cellular antigens and their corresponding antisera, with given receptor specificity, was only partially successful.

Previous findings from our laboratory (7, 8), however, provided an additional approach whereby an antiserum with specificity toward the group B coxsackievirus receptors of HeLa cells could be obtained. These findings showed that HeLa cells whose receptors were blocked by saturating levels of one member of coxsackievirus group B were unable to attach other members within the same virus group, whereas viruses of heterologous groups attached normally. Similar results were also obtained for the polioviruses. Thus, we reasoned that, to obtain an ACS with reactivity highly specific for the receptors which had been blocked by cell-bound virus, virus-saturated cells could be used as a cell antigen preparation for absorbing ACS prepared against normal cells. Two batches of HeLa cells were treated separately with chymotrypsin and saturating levels of coxsackievirus type B3 and then were used successively, as antigen, to absorb ACS prepared against normal HeLa cells. Conversely, HeLa cells which were saturated with poliovirus type 2 were used as an absorbent to obtain antiserum with specificity toward the poliovirus receptors. From the results presented in Table 6, it can be seen that the cells treated successively with chymotrypsin and coxsackievirus type B3 effectively reduced the anti-

TABLE 6. *Absorption from anticellular serum of antibody to receptors for assorted enteroviruses by HeLa cell-antigen preparations*

Treatment of cells for ACS absorption	Titer of ACS found to inhibit attachment of			
	Poliovirus T1	Coxsackievirus B1	Coxsackievirus B3	Echovirus T6
Chymotrypsin	<100[a]	ND[b]	1,200	ND
Trypsin	<100	ND	400	ND
Poliovirus T2	<50	300	ND	400
Untreated (absorbed once)	<100	ND	200	ND
Chymotrypsin and coxsackievirus B3[c]	<50	1,200	1,100	150
Untreated (absorbed twice)	<50	<50	<50	<50
ACS (unabsorbed)	200	1,200	1,600	3,200

[a] All numbers preceded by < refer to the results found for the lowest serum dilution tested.
[b] ND, Not done.
[c] ACS absorbed first with chymotrypsin-treated cells, followed by a second absorption with coxsackievirus B3-saturated cells.

body levels in the ACS to the poliovirus type 1 and echovirus type 6 receptors, while leaving behind the antibodies with reactivity for receptors for coxsackievirus type B1 and B3. On the other hand, it was surprising that the cells saturated with poliovirus type 2 maintained not only the capacity to absorb from ACS those antibodies directed to the receptors for viruses of heterologous groups, but also those antibodies reactive with the poliovirus type 1 receptors. This latter finding, which remains to be explained, is consistent, however, with those obtained when trypsin-treated cells were used as antigen either to absorb ACS or to immunize rabbits.

DISCUSSION

Anticellular serum has been shown to be effective in inhibiting attachment to the receptors of live HeLa cells of all of the enteroviruses tested, including members of coxsackievirus group B, which prior to this time had been considered refractory to inhibition by ACS. The ability of ACS to inhibit attachment of virus to cell receptors was found to be independent of complement activity. The cytotoxic titer of ACS, which had been absorbed successively with chymotrypsin-treated cells and by cells saturated with coxsackievirus type B3, was reduced from 1:800 to 1:50 when tested in the presence of complement, without a corresponding reduction in its inhibitory activity toward coxsackievirus type B1 receptors. Although this observation suggests that the coxsackievirus group B receptors are not the antigens involved in the cytotoxic reaction, additional studies are needed to evaluate this issue and to explore the relationship of viral receptors to other cellular antigens (6, 29).

A method has been described for the preparation of an ACS with a high degree of specificity for the group B coxsackievirus receptors of HeLa cells. Our inability to prepare an antiserum with specific reactivity for poliovirus receptors of HeLa cells, however, was unexpected. We consider it unlikely that the rapid rate of regeneration of poliovirus receptors after trypsin-treatment of HeLa cells (16) could account for the observed antigenicity of these inactivated receptors, since precautions were taken to avoid receptor regeneration by keeping the cells at 4 C in BSS. Further studies are necessary to determine the relationship between the virus binding site and that part of the cell surface which directs the formation of antibodies inhibitory to poliovirus receptors.

The studies reported in this paper have shown ACS to be capable of inhibiting attachment of enteroviruses to susceptible cells, without causing a corresponding inhibition of plaque formation by the same viruses. On the surface, these findings appeared to be incongruous. However, these results were shown to be reconcilable when the methodology inherent in the two forms of assay was evaluated. For inhibition of virus attachment, the titer of ACS was found to depend primarily upon the number of cells being treated, whether the cells were tested in suspension or in monolayers. The same relationship should also hold for inhibition of plaque formation by ACS. Thus, to obtain a degree of receptor blockade by ACS sufficient to be reflected in a reduction in plaque formation, a relatively high concentration of ACS is required. It should be emphasized that the standard assay used to titrate the activity of ACS for inhibiting virus attachment employed 2×10^6

cells per ml of serum, whereas in titrations of activity of ACS for inhibiting plaque production, 2×10^7 cells per ml of serum were employed. When the 10-fold difference in cell concentration used in the two systems of assay is considered, together with the knowledge that almost total blockade of receptors would be required to inhibit plaque formation, a plausible explanation can be provided for the difference found between the respective titers of ACS for inhibiting virus attachment and plaque production. These data demonstrate that, under the standard conditions of assay of ACS activity, inhibition of virus attachment is a more sensitive measure of ACS interaction with cells than is inhibition of plaque formation.

We anticipate that the results of these studies will provide a basis for the antigenic analysis of viral receptors of host cells. In addition, the availability of receptor-specific antiserum should aid in the identification of receptors, or of their subunits, after they are chemically extracted from cell membranes (17, 21).

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