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Adherence of Group B Streptococci and Human Erythrocytes to Influenza A Virus-Infected MDCK Cells (40424)

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Recently we formulated a hypothesis to explain the commonly observed association between influenza A virus infection and subsequent bacterial superinfection (1). The hypothesis states that cells infected with certain viruses may permit adherence of certain bacteria; this initial step may lead to bacterial colonization, infection and, in some cases, disease. The hypothesis was tested in a cell culture system. Strains representing eighteen species of bacteria were examined for their capability to adhere to canine kidney cells infected with influenza A virus. Only two species of the bacteria tested, both streptococci, adhered to cells in virus-infected cultures, but not to cells in control cultures. Pretreating the virus-infected monolayers with mouse ascitic fluid containing antibodies against influenza A virus completely blocked adherence of the streptococci—group B streptococcus types Ia, Ic and II, and *Streptococcus sp.* Thus, the phenomenon appears to be mediated by virus-induced receptors on the surface membrane of kidney cells.

The purpose of this study was to determine whether or not streptococci adhere to cells in influenza A virus-infected monolayers in a manner similar to adsorption of erythrocytes to the virus-infected cells, a phenomenon initially described by Shelokov *et al.* (2) and referred to as hemadsorption (HAd). The present experiments utilized scanning electron microscopy (SEM) to provide detailed information on the interaction of streptococci and erythrocytes with the surface of virus-infected cell cultures. The nature of receptor sites on the streptococci was compared with those on erythrocytes by pretreating the cells with receptor destroying enzyme (RDE) and studying the effect on bacterial adherence (BAd) and HAd, respectively.

Materials and methods. Cell cultures and virus inoculation. MDCK (stable canine kidney) cell monolayers were grown in aluminum foil plates (heavy duty, Kaiser Aluminum and Chemical Corp., Oakland, CA) after the method of White and McManus (3), and in 60 mm cell culture plates. Each culture contained 2 ml of Eagle's basal medium (Auto-Pow BME, Flow Laboratories, Rockville, MD) with 10% fetal calf serum, 0.03% glutamine, and neomycin (20 μg/ml) and was incubated in 5% CO_2 at 37°. Monolayers of MDCK cells were infected with influenza A/NWS/33 virus (a neurotropic variant of the Wilson Smith strain) as previously described (1). Control monolayers were sham inoculated with medium. Infectivity of virus-infected monolayers was determined by the HAd plaque assay (4).

Bacteria. Test bacteria included group B streptococci of types Ia (strain 090), Ic (strain A909), and II (strain 18RS21) obtained from Dr. H. W. Wilkinson (CDC, Atlanta, GA). Stock cultures were prepared as reported earlier (1).

Test system for bacterial adherence. Details of the test system have previously been reported (1). Briefly, influenza A virus-infected and control MDCK monolayers were washed with Hanks' balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, NY) without sodium bicarbonate, serum, or antibiotics. Fresh subcultures of the streptococci were washed and suspended in HBSS (10^9 organisms per ml). A 0.7 ml inoculum of bacteria was layered over the monolayers and incubated at 22–25° for 1 hr. Bacterial suspensions were aspirated from each plate and the monolayers were washed three times with HBSS. After applying a cover glass, each monolayer was examined using a fluorescence microscope equipped with a darkfield condenser and conventional light source. Virus-infected and control monolayers were also gram-stained. Results of BAd tests on

monolayers grown in aluminum foil plates were determined after the monolayers were fixed and prepared for SEM.

Hemadsorption. Washed human erythrocytes (HE) type O were used at a concentration of 0.4% in HBSS. In each adherence test, 0.7 ml of HE suspension was added to both virus-infected and control monolayers which were processed and examined microscopically as described for the streptococcus-treated cell cultures.

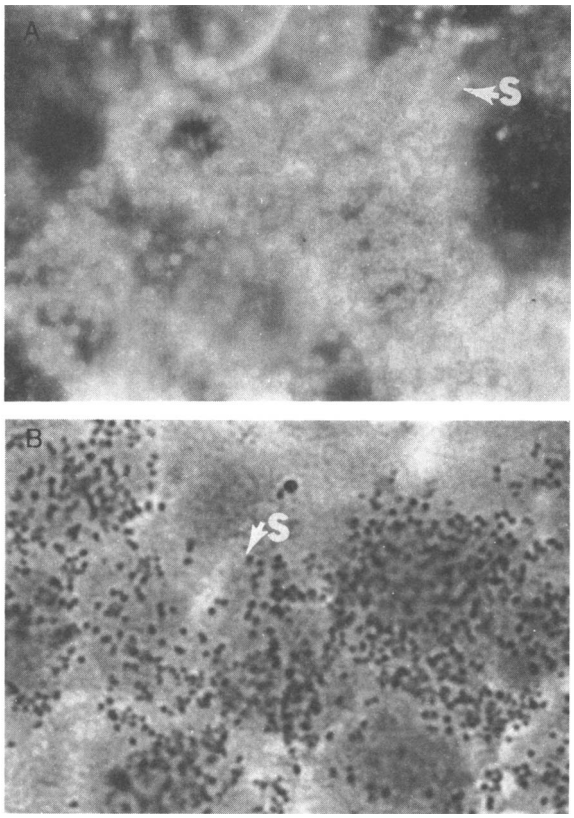
Preparation of specimens for SEM. Virus-infected and control monolayers, grown in aluminum foil culture plates, were exposed to suspensions of bacteria, HE, or an equal mixture of both for 1 hr as described. After washing, the monolayers were fixed *in situ* on the foil (5) by covering with 0.1% glutaraldehyde in HBSS, which, after 1 hr, was replaced with a 2% solution for an additional hour. During these and subsequent steps, care was taken to keep the surface of the monolayers in the plates under liquid to prevent cellular distortion. After fixation the monolayers were dehydrated in serial changes of 30, 50, 70, 95, and 100% ethanol. A circular disk (15 mm in diam) was cut out of each cell culture plate and critical-point dried by replacing the ethanol with liquid CO_2 in a Bomar SPC-900/EX critical point dryer (Bomar Co., Tacoma, WA). The foil disk was then mounted on an aluminum stub (15 mm × 5 mm; Structure Probe, Inc., West Chester, PA) with television tube koat (GC Electronics, Rockford, IL). Monolayers on the disks were coated with a layer, approximately 20 nm thick, of gold and palladium in a Model 200 Mini-Coater (Commonwealth Scientific, Alexandria, VA) and examined with a JEOL JSM-35U scanning electron microscope at 35–39 KV accelerating voltage. Stereoscopic pairs were photographed with a 6° difference in specimen tilt and observed with a fixed focus stereoscope viewer (Abrams Instrument Corp., Lansing, MI). Photographs were taken with a Polaroid 545 Land film holder using Polaroid Type 55 photographic film.

Bacterial adherence and hemadsorption blocking tests. Suspensions of streptococci used in the blocking tests were inactivated by mercury arc irradiation (2,000 $\mu w/cm^2$ for 5 min at 22–25°) according to the method of Wiley and Wilson (6). Washed virus-infected monolayers were overlaid with a 1 ml suspension of streptococci and incubated at 22–25° for 1 hr. Monolayers were then washed three times with HBSS and overlaid with a 1 ml suspension of HE at 22–25° for 1 hr. After three more washes with HBSS, monolayers were examined for BAd and HAd. A similar experiment was performed in which monolayers were exposed first to HE, then to streptococci.

Enzyme treatment of bacteria and erythrocytes. Streptococci were washed and resuspended in 6 ml of phosphate buffered saline (PBS; pH 7.2) to give a turbidity reading of 2.20 at 620 nm. Three ml of each strain of streptococci were inactivated by mercury arc irradiation. The two suspensions, containing either viable or UV-inactivated bacteria, were divided into 1 ml aliquots, pelleted, and resuspended in 5 ml of either PBS, calcium acetate saline (CAS; pH 6.2), or receptor destroying enzyme (RDE; *V. cholerae,* strain 4Z, CDC, Atlanta, GA), diluted in CAS (10 U enzyme/ml). Washed HE were also suspended in each of the three reagents (0.25 ml packed cells/tube). All cell suspensions were incubated at 37° for 2 hr according to the method of Ada and Stone (7). The bacteria and HE were washed twice in HBSS, resuspended in 1 ml HBSS, and used in the BAd and HAd tests, respectively, as described.

Results. Streptococcal adherence to virus-infected cells. Group B streptococci (types Ia, Ic and II; 7×10^7 bacteria/suspension) were added to virus-infected and control MDCK cell monolayers and the preparations were examined by darkfield, light, and SEM. Virus-infected monolayers were used 24–48 hr post-virus inoculation when the monolayers had $2–4 \times 10^3$ HAd plaques/plate. As seen in Figs. 1A and B, streptococci adhered to most cells in the virus-infected monolayers; they did not adhere to any cells in the control monolayers (not shown). The scanning electron micrograph of another inoculated culture in Fig. 2 shows that the cocci did not adhere to every kidney cell in the monolayer; presumably they adhered only to the virus-infected cells.

Results of blocking tests. The mean number of UV-inactivated streptococci adhering to virus-infected monolayers was determined in monolayers exposed only to bacteria (6×10^8



FIGS. 1–7. Microscopic studies of bacterial adherence and hemadsorption reactions. MDCK cell monolayers infected with influenza A virus were exposed to a suspension of streptococci (s) or to a suspension of streptococci mixed with human erythrocytes (e). Group B streptococcus type Ia was the strain used.

FIG. 1. Adherence of streptococci. A. Unstained specimen (darkfield × 1000). B. Gram-stained specimen (× 1000).

streptococci/plate) or exposed first to HE (3×10^7 cells/plate) then to bacteria. Each preparation was examined by darkfield microscopy and the mean number of bacteria adhering to 25 kidney cells was calculated. Pretreatment with HE decreased BAd from 71 cocci/kidney cell (range 41–127) to 6 cocci/kidney cell (range 3–10); bacteria adhered only to HAd-positive cells. A similar experiment was done to test the ability of streptococci to block HAd. Pretreatment of virus-infected monolayers with streptococci reduced the mean number of adsorbed HE from 34 erythrocytes/kidney cell (range 20–50) to 6 erythrocytes/kidney cell (range 2–18). Results of the blocking tests indicated that streptococci adhere only to virus-infected HAd-positive cells. In the next experiment we did a mixed BAd-HAd test in which virus-infected and control monolayers were exposed to a 0.7 ml suspension containing 10^7 HE and 3.5×10^7 streptococci (type Ia, Ic, or II). The preparations were examined by SEM and a representative field is seen in Fig. 3. The cocci are adhering to the HAd-positive MDCK cells while an HAd-negative cell in the foreground is free of adherent cocci, and presumably was not infected by the virus.

Microscopic details of adherence. SEM was used to examine the interface between kidney cells and adhering streptococci or HE. Figure 4 shows the streptococci adhering either to the surface membrane of the kidney cell or its filamentous projections. The stereoscopic image of a similar field in Fig. 5 helps visualize the three-dimensional interface between kidney cells and streptococci. The field in Fig. 6A shows a pair of adhering HE and

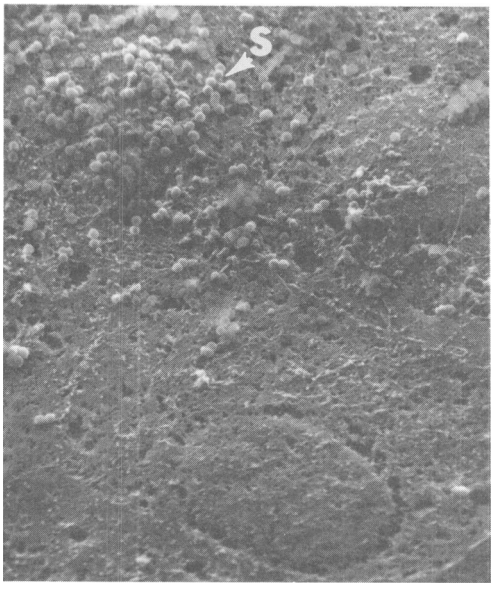


FIG. 2. Adherence of streptococci. Note that cocci adhere to some, but not all MDCK cells. Scanning electron micrograph (× 2400).

many cocci. A higher magnification (Fig. 6B) shows that the HE is attached to a long, thin filament apparently projecting from the kidney cell surface membrane. Also, at least two spherical particles, approximately 130 nm in diameter, marked by arrow heads, can be seen on the filament. (Our SEM preparations frequently showed such particles and also filaments on the surface of the HE.) The stereoscopic image in Fig. 7 shows that a filamentous projection can have receptor sites for both streptococci and HE. The filamentous projections from the surface of virus-infected kidney cells were consistently thinner and longer (average 77 nm × 1334 nm) than the microvilli commonly observed on the surface of control cells (average 114 nm × 200 nm).

Results of pretreating streptococci and erythrocytes with RDE. As seen in Table I, pretreating group B streptococci types Ia and Ic and HE with RDE destroyed the ability of the cells to adhere to virus-infected MDCK cell monolayers; enzyme treatment had no effect on the adherence of type II streptococci. Identical results were obtained in three separate experiments performed on different days; duplicate suspensions of both viable and UV-inactivated streptococci were used in each experiment.

Discussion. Previously we established that streptococci adhere to cell monolayers inoculated with influenza virus and that the cul-

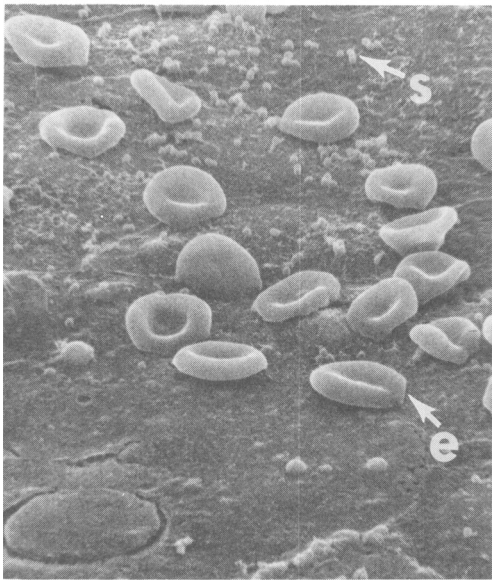


FIG. 3. Mixed bacterial adherence and hemadsorption reactions. Streptococci and erythrocytes are seen attached to the surface of the *same* kidney cells. Kidney cells devoid of bacteria and erythrocytes are also seen (upper right and lower left). The large kidney cell in the foreground has several "blebs" on its surface (× 1500).

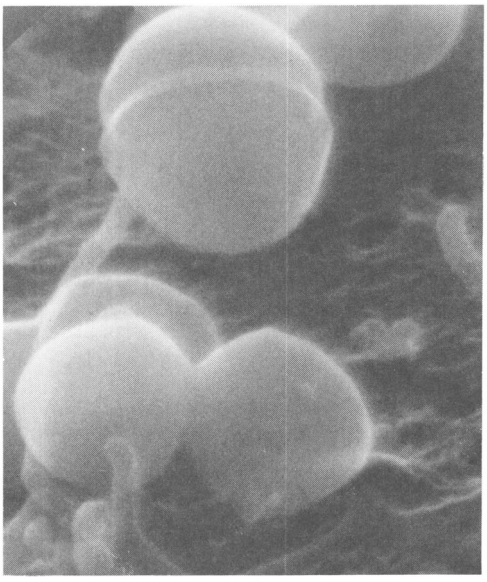


FIG. 4. Adherence of streptococci. Of the several adhering cocci in the field, one septate coccus is attached to a filamentous projection (× 54,000).

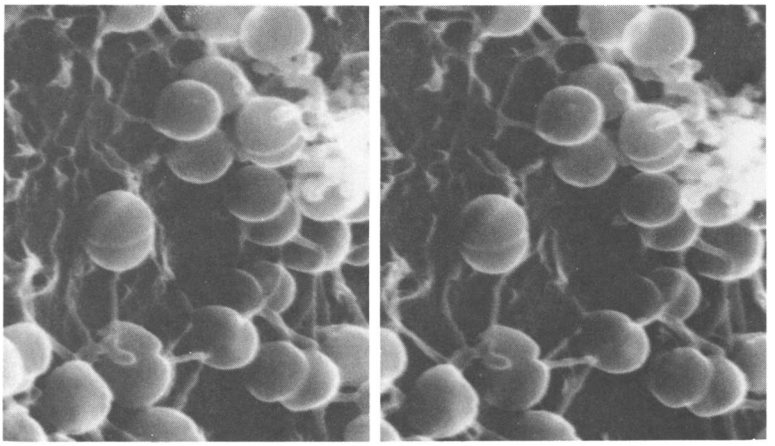


FIG. 5. Adherence of streptococci. Stereoscopic image photographed with a difference of 6° in specimen tilt. Note the network of filamentous projections; cocci are attached to some (× 16,000). (To obtain a three-dimensional image, observe with a stereoscopic viewer.)

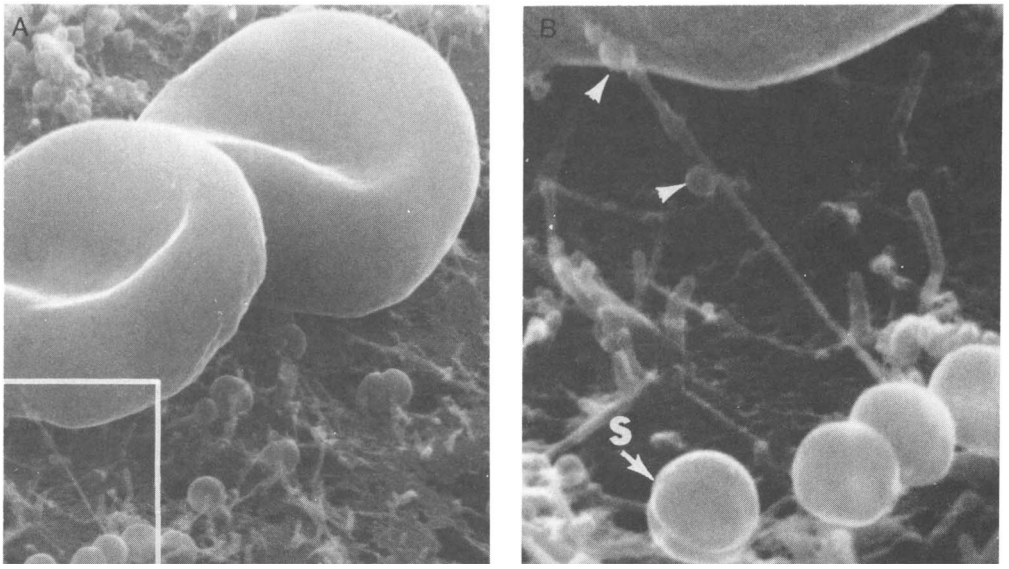


FIG. 6. A. Mixed bacterial adherence and hemadsorption (× 10,000). B. Higher magnification of the marked area in A, showing an erythrocyte adhering to a filamentous projection; particles resembling virions are seen on the side and tip of the filament (arrow heads). Cocci are seen in the foreground (× 30,000).

tures which were positive by bacterial adherence (BAd) were also positive by hemadsorption, HAd (1). In this study we compared the manner of attachment of human erythrocytes (HE) and group B streptococci to virus-infected MDCK cell monolayers. Using dark-field, light, and SEM we showed that the streptococci adhered only to HAd-positive MDCK cells: pretreatment of monolayers with HE decreased the numbers of adherent streptococci by 92%; pretreatment of monolayers with the streptococci decreased the numbers of adherent HE by 82%. Finally, in mixed BAd-HAd tests, the bacteria and HE were observed adhering to the same MDCK cells (Fig. 3).

The surface membrane of HE possess a glycoprotein receptor which combines with

influenza viruses resulting in viral hemagglutination, HA (8). This receptor is also responsible for the adsorption of HE to virus hemagglutinins which gradually appear over the entire surface of host cells infected with influenza virus (9–11). The hemagglutinins can be detected on filamentous projections of the cell surface which, in the case of influenza virus-infected cells, appear to be budding forms of the virus (12–16). Our SEM studies revealed two kinds of attachment of the bacteria and HE to virus-infected cells—either directly to the kidney cell surface or to the filamentous projections (Figs. 4–7). Both kinds of attachment of HE to monkey kidney cells infected with influenza A virus were described by Hotchin *et al.* (17) and Doane and Anderson (18). Our SEM results indicated a similarity in the attachment of bacteria and HE to virus-induced receptors on the surface of infected cells which, in the case of HE, are known to be hemagglutinins. Thus, it was important to determine if the viral hemagglutinins were also the receptors responsible for adherence of streptococci. Burnet and Stone (19) showed that the pretreatment of HE with receptor destroying enzyme (RDE) destroyed the HE glycoprotein receptor for virus hemagglutinins; the enzyme acts by liberating sialic acid from the intact HE (20). As expected, pretreating HE with RDE destroyed the capability of the cells to adhere to virus-infected MDCK cells (Table I). The obvious next step was to determine the effect of RDE-treatment of the test strains of group B streptococci types Ia, Ic, and II. Baker and Kasper (21) showed that sialic acid is a component of the type-specific antigens present on the cell walls of all five types (Ia, Ib, Ic, II, and III) of group B streptococci; they included the three specific strains used in the present study. Pretreating streptococci types Ia and Ic with RDE had the same effect as seen with enzyme-treated HE; the cocci were no longer able to adhere to virus-infected cells (Table I). These results suggest that HE and streptococci types Ia and Ic attach to infected cells by a similar mechanism. Surprisingly, the

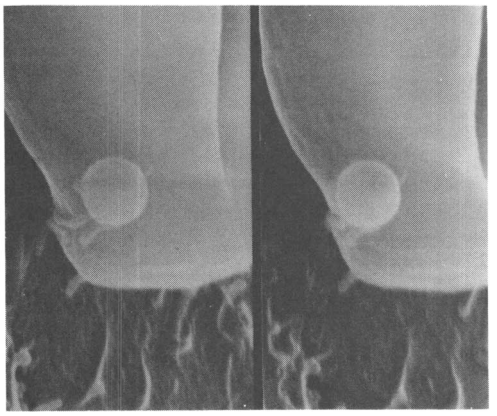


FIG. 7. Mixed bacterial adherence and hemadsorption. The stereoscopic image shows a coccus and part of an erythrocyte; both are attached to a tortuous, filamentous projection (× 16,000).

TABLE I. INHIBITION OF BACTERIAL ADHERENCE OF GROUP B STREPTOCOCCI AND OF HEMADSORPTION OF TYPE O HUMAN ERYTHROCYTES TO INFLUENZA A/NWS/33 VIRUS-INFECTED CANINE KIDNEY (MDCK) CELL CULTURES BY PRETREATMENT OF ERYTHROCYTES OR STREPTOCOCCI WITH RECEPTOR DESTROYING ENZYME (RDE).

	Bacterial adherence[b]							
	Virus-infected cells			Uninfected cells			Hemadsorption	
	Serotype			Serotype				
Pretreatment[a]	Ia	Ic	II	Ia	Ic	II	Virus-infected cells	Uninfected cells
PBS	+	+	+	−	−	−	+	−
CAS	+	+	+	−	−	−	+	−
RDE (50 U) diluted in CAS	−	−	+	−	−	−	−	−

NOTE: Cell cultures were inoculated with virus 24 hr before testing. (−) = no adherence or hemadsorption; (+) = adherence or hemadsorption.

[a] After washing, bacteria or erythrocytes were treated with 5 ml of the indicated solution for 2 hr at 37°. Cells were washed after treatment and before adding to virus-infected monolayers. PBS = phosphate buffered saline, pH 7.2; CAS = calcium acetate saline, pH 6.2; RDE = receptor destroying enzyme of *Vibrio cholerae*.

[b] No difference in adherence patterns were observed between viable and UV-inactivated suspensions of streptococci.

enzyme treatment did not inhibit adherence of type II streptococci. It is possible that sialic acid is not a part of the receptors on the surface of type II streptococci which enable the organisms to adhere to infected cells. However, it is also possible that the α-ketosidic linkage of sialic acid is not accessible to RDE or, if it is accessible, that it is not susceptible to hydrolysis by this enzyme under the experimental conditions used (22).

Summary. Scanning electron microscopy was used to compare the adherence of group B streptococci (types Ia, Ic, and II) and human erythrocytes to canine kidney cell monolayers inoculated 24–48 hr earlier with influenza A/NWS/33 virus. Streptococci and erythrocytes adhered to the kidney cell surface membranes and filamentous projections of some but not all the cells in virus-inoculated monolayers, while they did not adhere to control monolayers. Streptococcal adherence was blocked when the virus-infected cells were first exposed to erythrocytes. Likewise, hemadsorption was blocked when the virus-infected cells were first exposed to streptococci. In mixed bacterial adherence-hemadsorption tests bacteria adhered only to hemadsorption-positive virus-infected cells. Adherence of streptococci types Ia and Ic and erythrocytes was inhibited when the streptococci and erythrocytes were pretreated with receptor destroying enzyme. However, enzyme treatment of type II streptococci did not affect their ability to adhere to virus-infected cells.

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