REACTIONS OF MONKEYS TO EXPERIMENTAL MIXED INFLUENZA AND STREPTOCOCCUS INFECTIONS*

AN ANALYSIS OF THE RELATIVE ROLES OF HUMORAL AND CELLULAR IMMUNITY, WITH THE DESCRIPTION OF AN INTERCURRENT NEPHRITIC SYNDROME

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The high incidence and mortality from hemolytic streptococcus pneumonia which accompanied the epidemics of influenza during World War I are well known. The synergistic role of a virus plus a bacterial agent resulting in a severe specific respiratory disease was first demonstrated by Shope in 1931 (1) as a naturally occurring and experimentally reproducible phenomenon in swine influenza. Brightman (2) then observed some of the reactions to be unusually severe in ferrets receiving serial transmission of the virus of influenza. Subsequent culture of the lungs and heart blood revealed a concurrent invasion with a group C hemolytic streptococcus. In 1941 Glover (3) reported that ferrets became infected following intranasal inoculation of Streptococcus hemolyticus, group C, only when inoculated simultaneously, or a very few days earlier, with influenza virus. The infection with the combined agents was severe. In the same year, Schwab, Blubaugh, and Woolpert (4) reported preliminary studies from this laboratory showing a shorter survival period and a higher mortality rate in mice receiving mixtures of Streptococcus hemolyticus, group C, and influenza virus, type A, than in colony mates exposed to either agent alone.

Comparatively few observations have been recorded of the successful experimental induction of respiratory infections in the monkey by means of the hemolytic streptococcus or influenza virus, and, to our knowledge, no studies of mixed infections with these agents have been reported in monkeys, other than the preliminary observations from this laboratory (5, 6). Earlier reports (7-10) have dealt with the response of Macaca mulatta to intranasal inoculation with influenza virus A and with hemolytic streptococcus, group C, respectively. The experiments now presented are concerned with the reactions of monkeys to these agents administered either simultaneously or in varying sequence.

Materials and Methods

The preparation and the administration of inocula were carried out as previously described (9, 10). When the two agents, viz. Streptococcus hemolyticus, group C, and influenza virus,
type A, strain PR8, were given simultaneously, the total dosages were combined in a 3 cc.
mixture. Symptomatic, hematologic, bacteriologic, and immunologic studies were made
according to the technics previously described.

EXPERIMENTAL

Simultaneous Inoculation with Influenza Virus A and Hemolytic Streptococci, Group C.
Experiment I.—Six monkeys (M 7, 8, 7-5, 8-4, 8-5, 8-7) each received intranasally a mixture
of equal volumes of the stock influenza virus and streptococcus suspension. Obvious mani-
festations of infection were noted in only one of the six animals,—a transitory febrile response
occurring within 24 hours in M 8. Twenty-four hours after inoculation all throat cultures
showed the presence of type-specific hemolytic streptococci, which disappeared spontaneously
in from 3 to 9 days thereafter in all cases except one (M 8-5), in which the streptococci remained
present throughout a 3 week period of daily observations.

The initial hematologic response in all six animals was similar to that observed following
inoculation of streptococcus alone (10). Within 24 hours there was a sharp temporary granu-
locytic leucocytosis, with subsequent neutrophilic peaks of lesser amplitude occurring over a
period of 8 to 12 days after inoculation. However, between the 11th and the 15th postinocu-
lation days, four of the six animals developed an absolute or relative granulopenia with a
reciprocal lymphocytosis, suggesting a delayed virus leucopenia. In one animal M 8-5 (Fig.
1) there was no appreciable leucocytic response until the 4th postinoculation day, when the
total white cells jumped from 11,200 to 46,000, the neutrophils from 6,200 to 31,000, and the lymphocytes from 4,000 to 11,000 per c. mm. Forty-eight hours later the neutrophils and lymphocytes had returned to normal limits and remained there until the 15th postinoculation day, when the neutrophils fell to leukopenic levels. The response in this animal suggested that the virus may have delayed the usual immediate leucocyte response to the streptococcus, as well as inducing an absolute leucopenia after the 15th day. In five of the six animals a rapid progressive decline in red cells and hemoglobin occurred during a period of 8 to 11 days, associated with a reticulocyte response which developed 7 to 10 days after inoculation. As the hemolytic streptococcus infection subsided, spontaneous recovery of red cells and hemoglobin to normal levels was observed.

In response to the virus, mouse-protective antibodies were formed by the 10th to 14th day. The opsonic index showed little change in the average number of bacteria engulfed per leucocyte other than a moderate transitory rise in M 8-5 and M 8-7 five days after infection. Precipitins specific for the streptococcus were present in low titer prior to inoculation in the sera of four of the six monkeys (M 7, 8, 8-5, 8-7). Only one of these (M 8-7) developed an increase in antibody titer which was observed 2 weeks after inoculation. Two of the monkeys (M 8-4, 7-5) had no precipitating antibody beforehand, but displayed a positive reaction with undiluted antigen 5 and 11 days after inoculation, respectively. Thus three of the six monkeys showed some specific precipitin reaction in 5 to 14 days after inoculation.

Antistreptolysin titers were increased in two of the six animals: from 10 to 200 units per cc. in 8 days, dropping to 100 units by the 29th day in M 7; from 10 to 125 units by the 8th and to 400 units by the 29th day after inoculation in M 8.

Virus Inoculation Followed by Streptococcus Inoculation. Experiment II.—Seven monkeys were injected first with influenza virus and later with the streptococcus. Group I (M 1-5, 1-6, 1-7, 1-8) received the streptococci after 4 days and group II (M 2, 3, 4) 15 to 17 days after the instillation of virus.

In group I the inoculation of the streptococcus within 4 days of the previous virus instillation, produced obvious signs of infection in two of the four animals (M 1-7, 1-8). M 1-7, (Fig. 2) became pale, listless, and irritable within 48 hours after the streptococcus inoculation and remained in this state for a period of 7 to 8 days; fever was present for 6 days. M 1-8 showed similar signs on the 2nd day but had recovered by the 3rd day after the streptococcus inoculation. M 1-5 developed only a slight fever for 24 hours and M 1-6 presented no apparent manifestations of infection.

Significant hematologic responses were demonstrable in three of the four animals in group I. Two of these (M 1-5, 1-7) showed a typical virus granulopenia lasting 9 and 2 days respectively, after the influenza virus inoculation. In M 1-5, which showed a prolonged period of granulopenia, there was no leucocytic response following the streptococcus inoculation. M 1-7, however, developed a marked leucocytosis persisting for 15 days, during which time a rapidly progressive and profound hemolytic anemia also developed. One animal (M 1-6) showed no leucopenia following the virus, but a significant leucocytic response typical of a primary pyogenic invasion followed the streptococcus inoculation.

Continued observations of the monkeys in group I revealed new manifestations of reactivation of the infection in M 1-5 at a much later date coincident with the reappearance of streptococci in the nasopharynx. This animal developed signs highly suggestive of acute nephritis, the significance of which will be discussed in a subsequent section.

All of the monkeys in this group produced virus-neutralizing antibodies 8 to 9 days after virus inoculation. In response to the streptococci, no rise in the opsonic index was noted in any of the animals. Specific precipitins were evident in the sera of three (M 1-6, 1-8, 1-5) of the four animals by the 10th, 11th, and 15th postinoculation days, respectively. Antistreptolysin titers were increased in three of the four animals: M 1-5 showed a rise from 10 to
40 units per cc, on the 5th day, to 100 units on the 10th day, and to 1,000 units on the 15th day after inoculation; M 1–6 presented a slight rise from a normal level of 10 units to 30 units by the 5th day after infection; the titer of M 1–7 rose from a preinoculation level of 10 units to a maximum of 100 units on the 15th day after administration of the streptococcus.

Two of the three monkeys in group II, which received the streptococci 15 to 17 days after the virus, manifested evidence of infection. Both developed fever. M 4 exhibited pallor...
and weakness on the 4th and 5th postinoculation days. M 3 developed progressive weakness after the 3rd day, facial erysipelas on the 10th day, and died with streptococcus septicemia on the 15th day after inoculation. All three animals developed a definite granulocytopenia 3 to 4 days after virus inoculation, which persisted even following inoculation with the streptococci. It is noteworthy that M 3, which developed the fatal streptococcal septicemia was in a secondary leucopenic phase (9) at the time that the streptococci were introduced into the nasopharynx. Both M 3 and M 4 displayed virus-neutralizing antibodies by the 8th and 9th days, respectively, following virus inoculation. M 2 though surviving without symptoms, failed to produce any demonstrable antibodies. In response to the streptococcus, M 3 and M 4 both yielded specific precipitins by the 10th and 11th postinoculation days. The serum of M 2 which contained some precipitins prior to inoculation, nevertheless showed no increase in titer after infection. Antistreptolysin remained at the preinoculation level of less than 10 units per cc. in both M 2 and M 3, but the titer rose from 20 units to 175 units 11 days later in M 4.

**Injection of Inactivated Virus Followed by Inoculation with Active Virus and Streptococcus.**

**Experiment III.**—Earlier studies (9) have indicated that heat-inactivated virus may stimulate specific neutralizing antibodies when introduced intranasally. It was thought of interest, therefore, to repeat the sequence of living virus followed in 15 days by the streptococcus using monkeys previously "immunized" with heat-inactivated influenza virus. Thus, four animals (M 6-2, 6-3, 6-4, 6-5) were first inoculated intranasally with 3 cc. of virus inactivated at 70° C for 90 minutes. Fifteen days later 3 cc. of living stock virus were instilled into the nares, and after another 15 day interval a 3 cc. inoculum of streptococci was administered.

The peripheral blood equilibrium was not altered by the instillation of inactivated virus. After inoculation with active virus, one animal (M 6-2) which showed no change in total white cells, developed a relative lymphocytosis. All four monkeys in this experiment showed a moderate granulocytic leucocytosis 24 to 48 hours after inoculation with streptococci in sharp contrast to the three animals, described earlier, which had received living virus followed by streptococci in a similar sequence, but failed to develop the typical granulocytic response to the streptococcus invasion. None of the animals in Experiment III developed anemia.

At the time of the inoculation with active virus, complete mouse-protective antibodies were present in the sera of M 6-3 and M 6-5 while the titers of M 6-2 and M 6-4 reached this level only after 5 and 9 days, respectively, following the instillation of active virus.

Initial opsonic index studies in monkeys 6-3, 6-4, and 6-5 showed uniformly low phagocytic activity for group C streptococci and only in M 6-4 was any increase noted (from 0 to 3.3 bacteria per neutrophilic granulocyte between the 4th and 5th days): subsequent indices returned to the preinoculation level. Specific precipitins were produced by two of the four animals within a 12 day period (M 6-2, 6-4). Antistreptolysin titers were affected only in M 6-4 (10 units to 33 units per cc. on the 12th day).

No obvious symptoms were noted in any of the animals following the introduction either of the inactivated or the living virus. Nasopharyngeal cultures from asymptomatic M 6-2 and M 6-3 became negative for streptococci 48 hours after inoculation. However, the other two monkeys developed fever within the first 24 hours; streptococci were recovered consistently from the nose and throat of each. The temperature of M 6-5 returned to normal on the 2nd day, but that of M 6-4 (Fig. 3) remained between 105.4 and 106.4°F. for more than a week. After a temporary period of symptomatic improvement this animal died with septicemia 25 days after receiving the streptococcus. Postmortem examination revealed multiple abscesses in the lungs, liver, and spleen. Both the abscessed and the non-abscessed portions of the vital organs contained pure cultures of the group C streptococci, as did the heart blood and pericardial fluid. This constituted the second fatality from super-imposed infection (see also M 3, Experiment II).

**Inoculation with Streptococcus Hemolyticus, Group C, Followed by Inoculation with Influenza Virus A.**  
**Experiment IV.**—Eleven monkeys were inoculated initially with specific strepto-
cocc...s and later with influenza virus. Group I, eight animals, (M 9, 1-0, 1-2, 1-3, 3-2, 3-3, 1-08, 1-46) received the virus 4 days, and group II, three animals, (M 1, 5, 6), 15 to 17 days after the streptococci.

Fig. 3. Fatal streptococcus septicemia in monkey receiving virus followed by streptococcus

Only two (M 1-2, 3-2) of the eight animals receiving streptococci followed in 4 days by virus instillation, evidenced any immediate signs of infection. M 1-2, which will be discussed subsequently, developed symptoms and signs characteristic of nephritis, beginning 3 days after the introduction of virus. Eight days after virus inoculation, M 3-2 showed signs of an acute respiratory infection with fever, anorexia, cough, and pulmonary rales. Hemolytic streptococci were present in abundance in the throats of both animals during these episodes. Forty-six days after the streptococcus infection, and 42 days after the superimposed virus inoculation, monkeys 9 and 1-0 developed a condition closely resembling that of M 1-2, the significance of which will be discussed later in this paper. The specific strain of hemolytic streptococci was recovered from the throats of both animals.

A characteristic granulocytic leucocytosis was observed 24 to 48 hours after streptococcus
inoculation in all of the eight animals in group I. This was abruptly and prematurely interrupted in seven of the eight monkeys, however, by a virus-induced leukopenia which lasted for 3 to 4 days. Following this temporary suppression of the granulocytosis, the spiking leukocytosis previously observed after uncomplicated streptococcus instillation again became dominant for another 3 to 12 days. An appreciable degree of hemolytic anemia also developed in all eight of these animals, becoming maximal in the majority by the 7th to 8th day after ad-

Fig. 4. Note acute anemic episode and leucocytosis following streptococcus inoculation, with temporary suppression of white cells by virus after primary inoculation. After reinoculation with streptococcus, no leucocytosis was observed but sharp increases in the precipitins and opsonic index occurred, which, in turn, were promptly depressed by virus instillation. Note progressive anemia and nephritic syndrome following the virus reinoculation.

ministration of the hemolytic streptococci, with a subsequent rapid spontaneous recovery of both red cells and hemoglobin to normal levels as other evidence of subsidence of the infection accumulated. In three monkeys, however, the hemoglobin remained abnormally low for 4 to 5 months after the initial streptococcus-induced anemia despite a return of red cells to normal levels within 2 to 4 weeks—apparently reflecting a deficiency in iron reserves in these individuals. Monkey 1-2 (Fig. 4) is representative of the reaction observed in the animals of this group. Twenty-four hours after streptococcus inoculation, the total white count had risen from 17,650 to 34,200 per c. mm. (neutrophils from 3,500 to 14,700) and remained abnormally high during the next 3 days. At the end of this 4 day period, influenza virus was
administered intranasally and within 48 hours, the total white cells fell from 24,600 to 14,750 (neutrophils from 14,760 to 10,470). Four days later the leucocytosis was again apparent (32,350 W.B.C.) and persisted through the 19th day of observation.

None of the animals showed any increase in polymorphonuclear phagocytic activity for these streptococci during this period. Specific precipitins were present prior to intranasal instillation of the streptococci in three of the eight monkeys (M 1–0, 3–2, 3–3), while 2 (M 9, 1–46) of the remaining five animals developed specific antibodies by the 4th day. Three monkeys (M 1–2, 1–3, 1–08) failed to produce any demonstrable precipitins. The precipitins were sharply reduced in the sera of three monkeys (M 3–2, 3–3, 1–46) following the administration of virus.

An increase in antistreptolysin titers was noted in only three of the eight animals in this group. Titrations for complement made daily on the serum of four animals in this series (M 3–2, 3–3, 1–08, 1–46) revealed no significant change in titer following either the streptococcus or virus inoculation.

A slight febrile reaction in M 1 and M 5, 24 to 48 hours after the administration of streptococci, was the only recognizable evidence of disease in the three animals comprising group II, Experiment IV, which received the streptococci initially followed by virus after an interval of 15 to 17 days. All three animals in this group, of which M 6 (Fig. 5) is representative, responded to the streptococcus instillation with a marked neutrophilic leucocytosis; virus administration 15 to 17 days later was followed in two of the animals by a typical leucopenia appearing within 1 to 3 days, apparently uninfluenced by and not influencing significantly, the previous streptococcus inoculation. The leucopenia in the third animal (M 1) was delayed, reaching slowly and progressively the lowest point on the 10th day after virus instillation.

Immunologically the monkeys responded with the production of specific precipitins by the 5th, 8th, and 28th days, respectively, after the streptococcus inoculation. Antistreptolysin determinations disclosed a rise in M 5 from 50 to 55 units per cc. by the 9th day and to 100 units on the 27th day after inoculation; titers in M 6 indicated an increase from 20 units prior to infection to 50 units 6 days later, and to 100 units 26 days after inoculation. Virus-neutralizing antibodies became demonstrable in all of these animals by the 14th day after introduction of the the virus.

Reinoculations with Streptococcus and Virus. Experiment V.—A series of animals was reinoculated with streptococci and/or virus 10 weeks to 9 months after primary inoculations with the same agents, to observe any possible differences in the response of a given monkey to initial versus reinfection.

Five monkeys (M 9, 1–0, 3–3, 1–08, 1–46) received streptococci followed in 4 days by virus, while two (M 1–6, 1–8) received virus followed, after the same interval, by streptococci. Four to seven months later each was reinoculated with the original strain of streptococci. A slight transient edema over the left eye of M 1–46, coincident with a transitory febrile reaction lasting 24 hours only, and a moderate fever between the 3rd and 5th days in monkeys 1–8 and 1–08 were the only obvious immediate evidences of infection in this group. Two animals (M 3–3, 1–08) developed a slight relative neutropenia after reinoculation with the streptococci; the white blood cell equilibrium in M 1–46 was not significantly altered. The anemia which had persisted in all three of these animals since the initial streptococcus inoculation 4 months earlier was not increased after reinoculation.

In vivid contrast to the lack of opsonic response to primary inoculation, the immune response to reinoculation in six of the monkeys in this group was dramatic and was characterized by a prompt and significant increase in the specific phagocytic properties of the circulating granulocytes. Preinoculation levels of 0 to 5 bacteria per cell were increased to averages of 9 to 18 per cell within the 1st week, and the opsonic index remained elevated during the follow-
ing 4 weeks of observation. Precipitins which were present in the serum of four of the seven monkeys did not increase appreciably following reinoculation. Antistreptolysin titers likewise were not significantly altered.

![Graph showing changes in temperature, throat culture, precipitins, antistreptolysin titre, opsoncytophagic test, virus neutralizing antibodies, platelets, reticulocytes, white blood cells, lymphocytes, and monocytes over time after initial inoculation.

Fig. 5. Marked neutrophilic leucocytosis developed after primary streptococcus inoculation with leucopenia following primary virus inoculation. Note increase in opsonic index after streptococcus reinoculation with sharp temporary 30 day suppression by the virus.

Three monkeys (M 1-2, 1-3, 1-7) received streptococci followed by virus after 4 days; 10 weeks after primary inoculation they were reinoculated with the original strain of streptococci and 19 days later received their second instillation of influenza virus. Two monkeys (M 1-2, 1-7) developed signs of infection. M 1-2 had presented transitory signs and symptoms of nephritis after primary inoculation with the streptococci followed by virus and still harbored streptococci in the throat prior to reinoculation. No immediate evidence of infection was apparent but after 2 weeks a slight edema of the ankles was noted. Three days after
virus inoculation the condition of the animal began to grow progressively worse. The edema and general malaise became more pronounced. Hemolytic streptococci were consistently recovered from the throat. One month after virus reinoculation the animal was sacrificed. Cultures made of the heart blood, spleen, liver, and kidneys, however, were negative.

Monkey 17, in contrast to M 1–2, was not a carrier of streptococci in the throat prior to reinoculation. Edema of the legs, ankles, and arms began to appear the 4th day following streptococcus reinoculation and became progressively worse, accompanied by fever. The streptococci disappeared from the throat by the 8th day. The subsequent administration of virus on the 19th day did not appear to aggravate the previous symptoms although a transitory rise in temperature occurred between the 3rd and 5th days following virus reinoculation. The edema decreased progressively and the animal recovered completely within the next month.

One monkey (M 6) was permitted an interval of 17 days between the initial streptococcus instillation and the subsequent virus inoculations. A 9 month interlude of apparently normal health followed before reinoculation with the original group C streptococci. No signs of disease having developed, a second reinstillation of virus was made after 19 days, which was also asymptomatic.

The white blood cells were not significantly altered in any of these four animals by the reinoculation of either the streptococcus or the virus. However, the red cells and hemoglobin levels fell with a resultant progressive anemia for 40 to 70 days after streptococcus reinoculation in all four monkeys. In monkeys 1–2 and 1–7 the appearance of the signs of nephritis was accompanied by an increase in the severity of the anemia.

In each of these animals the reinoculation of streptococci was characterized by a prompt and significant increase in the phagocytic index. Following the reinoculation of virus, however, the opsonins were depressed within 24 to 48 hours. M 6 (Fig. 5) showed a rise from a preinoculation level of 0.5 to 6 streptococci per phagocyte within 24 hours and to 15 per cell within 48 hours of group C reinoculation. The average remained between 15 and 35 bacteria per phagocyte until virus reinstillation, when the index of 23 dropped to 5.5 within 24 hours and remained between 0.5 and 6 cocci per cell for the next 3 weeks; after 1 month the index rose again slowly, 11 to 13 bacteria being counted per leucocyte. M 1–2 (Fig. 4) reacted similarly with an increase in opsonins within 48 hours after streptococcus reinoculation to a maximum of 11 organisms phagocytized per cell. This was maintained until the administration of virus, when there was a significant drop to 1 to 7 bacteria per cell for a month; 5 weeks later the opsonic index again rose to 26.5.

Precipitins were present prior to reinoculation in one (M 1–2) of the four monkeys. Following reinoculation with streptococci, precipitins were detected after 4 and 20 days, respectively, in M 1–3 and M 6. After instillation of the virus, no precipitins could be detected for 1 to 5 days in M 1–2 and M 1–3, but reappeared after the 5th day.

Antistreptolysin titers in M 1–7 rose from a baseline of 10 units per cc. prior to streptococcus reinoculation to 100 units per cc. 5 to 10 days afterwards; the day following virus reinoculation the titer again dropped to 10 units, then 5 days later rose to 100 units. M 1–2 and M 6 showed a delayed rise from 10 to 100 units 27 and 53 days, respectively, after streptococcus reinoculation.

In summary the following general observations were made in monkeys reinoculated at intervals varying from 10 weeks to 9 months with the streptococcus and/or the influenza virus:

1. Two of thirteen monkeys initially infected with streptococci and virus, after reinoculation with the same strain of streptococcus, developed persistent signs and symptoms suggestive of nephritis (see next section).
2. The opsonic index, reflected by the specific phagocytic activity of the granulocytes of the monkeys' blood, which was slightly higher than it had been previous to and within several weeks after primary inoculation, displayed a distinct and significant rise in twelve of thirteen animals following reinoculation with streptococci; the increase appeared within 24 hours in five animals; in four the opsonins rose within 3 to 6 days; in the remaining three animals a significant increase occurred during the 2nd week. In the monkeys receiving virus after the streptococcus reinoculation, there was a sharp depression of these specific phagocytic properties, frequently within 24 hours, and followed by progressive anemia reflecting in all probability the released hemolytic activity of the streptococci.

3. Precipitins were present in six of thirteen monkeys following primary streptococcus inoculation and prior to reinoculation. A rise in titer was demonstrable following reinoculation in four monkeys and in only two of the seven lacking precipitins did any detectable antibody development occur after reinoculation. Following the administration of virus the precipitin level was lowered in two of three animals having demonstrable antibodies of this type.

4. Specific antistreptolysin titers were increased by streptococcus reinoculation in three of the ten animals tested and were neutralized by virus instillation temporarily.

An Acute Nephritic Syndrome

During the preceding studies five monkeys developed signs and laboratory findings strongly suggestive of acute nephritis. Three (M 9, 1-0, 1-2) of the five received streptococci followed by virus within 4 days, and two (M 1-5, 1-7) were first given virus followed in 4 days by streptococci. Monkey 1-2 (Fig. 4) which received streptococci intranasally followed by influenza virus 4 days later, showed edema of the soft tissues around the eyes within 7 days of the streptococcus inoculation. Two days later marked edema of the ankles and legs developed. At this time the urine contained 100 mg. per cent of albumin and red cells (260 per c. mm.). The albumin values varied between 35 and 100 mg. per cent for the next 8 days during which time the edema gradually disappeared. Throat cultures throughout this period were consistently positive for hemolytic streptococci, group C. Ten weeks later this monkey was reinoculated with the same strain of hemolytic streptococcus followed by the same strain of virus in 17 days. On the 14th day after streptococcus reinoculation the animal began to display generalized edema, and on the 19th day (2 days after virus) appeared weak, pale, and listless. Blood pressures taken before reinoculation fluctuated between systolic 180 to 140, diastolic 90 to 110 mm. Hg. Coincident with the appearance of the above signs, the blood pressure readings increased to systolic 185, diastolic 140 mm. Hg, and the hematuria was persistent. Forty-seven days after reinoculation with the streptococcus, the edema, although varying in degree, was still present. At this time the monkey was sacrificed. The kidneys were grossly congested and enlarged. Microscopically, the cortex showed areas of necrosis and disintegration of the epithelial cells of the convoluted tubules with areas of coagulation debris in the tubular spaces. There was one small focus noted which showed acute inflammation with numerous polymorphonuclear cells. The medulla presented a similar picture. The glomeruli showed some thickening of the capsular epithelium with occasional polymorphonuclear cell infiltration. Some sections showed few polymorphonuclear cells in the glomerular tufts.
Monkeys 9 and 1-0, which had received streptococci initially followed by virus in 4 days also exhibited signs suggestive of nephritis on the 46th day after inoculation with the streptococcus. In monkey 9 the edema lasted for 7 days while hypertension was first noted on the 4th day of illness when a systolic of 170 mm. and a diastolic of 120 mm. Hg were recorded in contrast to a baseline reading of 110/80 mm. Hg. The elevation in blood pressure was maintained for 12 days. Throat cultures were positive for Streptococcus hemolyticus, group C, throughout this period. Monkey 10, on the 46th day, developed marked generalized edema, and 4 days later a rise in blood pressure was noted from 110/90 to 140/110 mm. Hg which persisted for 4 days. The urine during this period showed many red blood cells and casts. This animal recovered spontaneously within a month after the onset of this syndrome.

Monkeys 1-5 and 1-7 received virus followed by the streptococcus 4 days later. Two months after inoculation, anasarca, pallor, albuminuria, and hematuria appeared in monkey 1-5. Throat cultures, as in each of the above animals, were positive for Streptococcus hemolyticus, group C. At this acute stage, monkey 1-5 was sacrificed. The kidneys were enlarged, markedly congested, and reddened. Microscopically, the glomerular tufts were thickened, congested, and there was sparse polymorphonuclear infiltration. There were occasional areas of interstitium, chronic inflammation with lymphocytes and polymorphonuclear cells present. Larger areas with large and small mononuclear cells, and a few polymorphonuclear cells were noted in apparently necrotic tubules, which also contained occasional smooth casts. The microscopic sections of the skin showed evidence of subcutaneous edema. Monkey 1-7 received a reinoculation with the same experimental strain of streptococci 3½ months after primary intranasal inoculation, and 17 days later was exposed to influenza virus A. One week after reinoculation with the streptococcus, a generalized edema appeared and increased to a maximum during the next 2 weeks, then gradually receded during the following 2 months. The blood pressure increased, coincident with appearance of the edema, reaching a maximum of 160/124 mm. Hg, returning to normal (110/80 mm.) after 3 weeks. Slight albuminuria was noted throughout this episode and throat cultures were positive for the hemolytic streptococcus, group C, for 8 days after reinoculation.

In summary, five monkeys in this series of studies showed signs suggestive of acute glomerular nephritis. One monkey (M 1-2) first exhibited these signs 6 days after initial experimental streptococcus infection and recovered spontaneously; reinoculation with the hemolytic streptococcus, group C, 10 weeks later was followed within 2 weeks by a second nephritic episode. Reinoculation with the experimental strain of streptococci 3½ months after primary inoculation similarly affected M 1-7 within the 1st week. The other three monkeys (M 9, 1-0, 1-5) developed edema, hypertension, and albuminuria 46 to 60 days after initial inoculation with the group C streptococci followed by virus, coincident with the reappearance of the specific strain of streptococci in the throat.

**DISCUSSION**

Previous studies describing the responses of monkeys to influenza virus (9) and to hemolytic streptococcus (10) infections, and the experiments here reported with these agents administered simultaneously or in varied sequence, have provided additional data bearing on the cellular and humoral factors concerned in resistance and upon the circumstances affecting the pathogenesis of the infections caused by these agents.
Normal rhesus monkeys remained relatively asymptomatic following the intranasal administration of the PR8 strain of influenza virus or the group C Streptococcus hemolyticus. These animals, however, developed characteristic peripheral blood cell and specific antibody reactions to these respective agents.

In response to living virus a granulocytopenia occurred between the 1st and 7th postinoculation days, followed by spontaneous recovery or in some cases by a relapsing (secondary) leucopenia. Virus-neutralizing antibodies appeared in the peripheral blood about the 8th postinoculation day. The coincidence of the recovery from the leucopenia with the appearance of antibodies suggests an interdependence of these factors, and this relationship is further emphasized by the fact that with reinoculation of the same virus, peripheral granulocytopenia did not follow. That the leucopenia does not occur, when, and because specific virus-neutralizing antibodies are present in sufficient titer, is indicated by the failure of active virus instillation to alter the peripheral white cell equilibrium after a previous inoculation with virus.

In sharp contrast to the relatively resistant state of normal healthy monkeys to intranasal inoculation with influenza virus, animals subjected to altered conditions of exposure and route of inoculation, and those in a nutritionally deficient state showed a significantly diminished resistance to this infection. Thus, all of four animals so exposed and subjected to lowered environmental temperatures exhibited symptoms and signs consistent with severe influenzal pneumonitis with three fatalities. Similarly, two of the four normal animals inoculated intratracheally demonstrated signs consistent with influenza, and five of seven nutritionally deficient monkeys failed to survive the virus infection. In contrast, ten normal monkeys which exhibited characteristic leucopenic and specific antibody evidences of virus invasion showed no other signs or symptoms of disease. The antibody responses were similar in time of appearance and titer in all animals receiving the virus, so that the differences in resistance to infection could not be ascribed solely to these factors. The specific virus-induced leucopenia, however, was more profound and prolonged in the deficient animals, being superimposed upon the nutritionally induced leucopenia already existing. This would favor secondary pyogenic invasion, and reduce any antiviral cellular activity. The fact that intratracheal inoculation, which bypasses the upper respiratory epithelium, produced influenzal pneumonitis, suggests that the upper respiratory epithelium may constitute an effective barrier against virus invasion of the lungs. The fact that monkeys with nutritional deficiency syndrome usually show gross lesions of the oral and gastrointestinal mucosa and abnormalities of the skin, suggests that qualitative changes in respiratory epithelial and other tissue barriers may be responsible for a quantitatively greater virus invasion under these circumstances. The precise mechanism by which exposure to cold increases susceptibility to virus invasion is obscure although the observation is consistent with common clinical experience.
in human beings. In these observations in monkeys it is to be noted that their body temperatures were lowered to levels approximating the optimal temperature for influenza virus propagation and, incidentally, to approximately the normal temperature of man, a more susceptible species.

The markedly diminished resistance of nutritionally deficient monkeys to hemolytic streptococcus infection was quite apparent (10). Whereas blood cultures taken from normal monkeys receiving streptococci were consistently negative, two of the five nutritionally deficient animals developed facial erysipelas and five of the six died with streptococcus septicemia. In evaluating this contrast in resistance of the normal and the nutritionally deficient animals, it must be noted that, as in the case of the influenza-infected animals, little difference was found in the humoral antibody responses of the two groups. There was a striking and significant difference, however, in the circulating cellular reactions. In the nutritionally deficient leucopenic animals, the granulocytic response was minimal and of brief duration, failing to attain even normal baseline levels in three of the six animals. Furthermore, the neutrophils, which did respond to this pyogenic stimulus, were qualitatively poor, containing multilobular nuclei, diminished numbers of toxic granules, and showed a tendency to cytoplasmic swelling and early fragmentation. In four of five animals the abortive leucocytosis was followed by a marked absolute leucopenia, which was definitely more pronounced than that observed in numerous animals with terminal nutritional leucopenia, but without superimposed infections. The anemia, too, which developed in these animals was characteristically more profound than that observed in nutritionally normal animals following streptococcal infections. The absence of an appreciable reticulocyte response in this nutritionally deficient group was in contrast to the response of normal animals to the hemolytic anemia, and in contrast also to other uninfected nutritionally deficient animals. In two animals there was a significant drop in thrombocytes following inoculation. Thus, the introduction of hemolytic streptococci, intranasally, in those monkeys showing a well established nutritional pancytopenia would seem to have superimposed a further toxic depressive factor on an already hypoplastic bone marrow.

The state of the bone marrow then, and hence the quality and quantity of the peripheral white blood cell response would appear to be a significant point of difference between the normal and the nutritionally deficient monkeys in their resistance to streptococcus infection. The marked immediate leucocytosis in normal monkeys following streptococcus infection and the gradual return to normal with the appearance of specific antibodies, reflect an adequate bone marrow potential for important assistance in disposing of the infectious agent. The abortive leucocyte response in nutritionally deficient monkeys may frequently fail to dispose of a superimposed infection. In addition to these factors, qualitative changes in the epithelial barriers effected by the nutritional
deficiency may play a part in streptococcus invasion similar to that suggested in connection with diminished resistance to virus invasion.

Obvious signs of serious infection were observed more often in our normal monkeys after receiving the experimental strain of *Streptococcus hemolyticus*, group C, superimposed upon a previous instillation of influenza virus A. In five of eleven monkeys, malaise, fever, anorexia, and general irritability appeared within 4 days. Three of these recovered spontaneously in 2 to 3 days, but the infections in two (M 3, 64) continued, terminating in a fatal septicemia 15 and 25 days, respectively, after the streptococcus inoculation.

When the order of administration of the infectious agents was reversed, *i.e.* with the streptococci preceding the virus, only one of eleven animals immediately became ill. Physical signs of nephritis appeared in this animal 3 days after virus inoculation. Other monkeys in this group, however, developed a delayed reaction 44 to 114 days postinfection, characterized by edema of the arms and legs, hypertension, and anorexia, coincident with the spontaneous reappearance of the *Streptococcus hemolyticus*, group C, in the nasopharynx of two, and after the intranasal reintroduction of this strain of streptococci in two others. Similar signs of delayed infection were observed in one monkey (M 1-5) 2 months after inoculation with a virus followed by streptococcus. Whether these signs, suggestive of renal involvement, were the result solely of the streptococcus infection or resulted from a combination of the two agents employed is difficult to determine precisely. The monkeys which developed these signs showed serum precipitins with undiluted antigen only, at a time when their throat cultures were positive, whereas the other animals which were inoculated with streptococci continued to show precipitins of higher titer after the specific organisms had disappeared from their throats. This suggests that the reappearance of streptococci in the throats of these animals, in the presence of transitory antibodies of low titer only, could have resulted in specific sensitization to the streptococcus antigens. It is to be noted further that of all the animals which reacquired the specific strain of streptococci, either spontaneously or by deliberate reinoculation, only those animals developed the nephritic syndrome, which had also received influenza virus at some time, always a specific depressant for streptococcus humoral antibodies and for granulocytosis under the conditions of these experiments.

The simultaneous administration of hemolytic streptococci and influenza virus usually resulted in both an immediate and a delayed hematologic response. The dominant, initial immediate leucocytosis reflected the invasion of streptococci, which masked the virus-induced leucopenia for some days. In one instance only, the virus acted more promptly to temporarily inhibit the streptococcus-induced leucocytosis. When virus preceded streptococcus inoculation by 4 days a "typical" leucocytosis was apparent in half the animals. When, however, the streptococci were not superimposed until 15 to 17 days
after the virus, the granulocytic leucocytosis was completely inhibited in all test animals. Apparently, therefore, this virus effect was more profound, so far as the leucocytic suppression was concerned, after 15 days than after 4 days. Noteworthy is the fact that this effect persisted after the usual period of recovery from primary virus leucopenia, which suggests that this virus inhibition of leucocytosis may perhaps be a factor in susceptibility to secondary pyogenic infections for a longer period than had been recognized previously. One-half of the animals in the 4 day sequence and two-thirds in the 15 day sequence ultimately became ill, with two fatalities.

In primary streptococcus inoculations many animals showed an hemolytic anemia which progressed only as long as there was active invasion, as reflected by the leucocytosis, and was followed by spontaneous recovery. Following reinoculation with hemolytic streptococci, the opsonic index was usually promptly elevated and no leucocytosis, no hemolytic anemia, and no other signs of infection developed, unless and until the virus was introduced. Thereupon, coincident with a sharp and sudden fall in the opsonins, a progressive hemolytic anemia without leucocytosis developed, and the occurrence of other signs of disease already mentioned (nephritic syndrome) reflected the release of streptococcic antigenic and hemolysin activity.

CONCLUSIONS

1. The vital importance of the cellular defense forces in the resistance of the monkey to combined streptococcus and influenza virus infections has been demonstrated.

2. Some of the conditions prejudicial to the maintenance of an optimum cellular reserve in the infected animal have been revealed; viz., undernutrition, physical cold, intratracheal route of infection.

3. The potential threat exerted by latent foci of streptococci, and the importance, in relation to the combined infection with virus, of cellular and humoral immunity, together or separately, have been demonstrated. The essential role of optimum nutrition (notably as concerns the vitamin B complex, and folic acid specifically) in the prevention of disastrous illness from these infectious agents, individually or in combination, would seem to have been proven.

4. Signs of glomerular nephritis appeared in a significant number of monkeys receiving Streptococcus hemolyticus and influenza virus in sequence, followed by reinoculation or spontaneous reappearance of the streptococci.

5. Reinoculation of Streptococcus hemolyticus, group C, resulted in a prompt "booster" increase in the opsonic index. Virus instillation was followed by just as sudden a depression in this index.

6. Reinoculation failed to evoke either the granulocytosis or the leucopenia in monkeys which are characteristic effects of the streptococcus and the virus
respectively when these agents are introduced for the first time by way of the nasal mucous membrane.

7. Simultaneous intranasal inoculation of influenza virus, type A, and *Streptococcus hemolyticus*, group C, in nutritionally normal *Macaca mulatta* failed to produce obvious signs of disease. In most of the animals, however, a streptococcus-induced leukocytosis followed by a delayed virus-induced granulopenia developed.

8. Inoculation of influenza virus followed in 4 to 17 days by streptococci produced obvious signs of disease in five of eleven animals which had become leukopenic as result of the action of the virus, and fatal streptococcal septicemia in two monkeys.

9. The development of signs of infection in previously healthy monkeys exposed to virus followed by streptococci confirms both the clinical and laboratory experience of other observers, that virus infection may predispose to secondary bacterial invasion, and, that at times, under unfavorable circumstances, the infection may become overwhelming. Although the complete mechanism of resistance is as yet not wholly clear, the depressant or inhibitory effect of the virus on both its cellular and humoral elements has been established.

**BIBLIOGRAPHY**