TABLE IV. Effect of Preincubation in Basal Medium on the Activity of Avidin.

Absence or presence of biotin during preincubation	Incubation menstruum	Activity of avidin ($\times 10^{-4}$ units) Avidin (NBC) Incubation (hr) 0	Avidin (NBC) Incubation (hr) 2	Avidin (GB) Incubation (hr) 0	Avidin (GB) Incubation (hr) 2
Absent	Distilled water	4.5	5.0	6.0	6.2
	Basal medium	2.2	1.5	2.2	1.5
Present	Distilled water	4.9	4.8	5.5	5.5
	Basal medium	4.9	4.8	5.5	5.5

Avidin (approximately 5×10^{-4} unit) was incubated with or without biotin (5.5×10^{-4} μg) in each steril menstruum.

with biotin(8). These ions, however, do not seem to act directly on the biotin binding site of avidin to cause the irreversible loss of activity, since avidin-biotin combination is not affected by the presence of the ions if the avidin and biotin are added simultaneously. The avidin-biotin complex thus formed is much more resistant to heat than that formed in the absence of the ions. The ionic agents might exert their effects by non-specifically upsetting the delicate charge balance of the protein resulting in an alteration of the protein structure so that the biotin binding sites of avidin become less susceptible to hydrolytic agents.

In view of the fact that all biological materials contain various ions which are capable of inhibiting the subsequent liberation of biotin from avidin, the prospect for the use of avidin as a tool in the purification of biotin appears to be discouraging. This is complicated further by the fact that commercial preparations of avidin contain significant amounts of biotin which will affect both the quantitative and qualitative recovery of the vitamin.

Summary. Data were presented on the quantitative release of biotin from avidin-biotin complexes formed under several conditions. The presence of various ions was found to interfere with the binding and release of biotin.

It is a pleasure to thank Miss Toby Kabakoff for excellent technical assistance in this work.

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Progressive Descending Influenza Infection in Mice Determined by Immunofluorescence. (29201)

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Hers(1) reviewed studies of influenza virus by immunofluorescence (IF) in tissue cultures and embryonating eggs and indicated that few studies have been made on pathogenesis of influenza by IF in adapted or natural hosts. Liu(2) studied human influenza

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virus infection in ferrets. He noted specific nuclear and cytoplasmic fluorescence in cells of nasal turbinates, bronchi, and alveoli by direct IF.

Hers *et al*(3) reported on the pathogenesis of S-15 virus in mouse lungs and followed the synthesis of S and V antigen by indirect IF. These studies and others have not followed the virus infection during the entire period of the disease nor has there been confirmation of the existing view that influenza is a descending type of infection. The following investigation reports the progression of S-15 virus in mice as it descends from bronchi to bronchioles to the alveoli.

Experimental animals. Pigs used for antibody production against S-15 virus were 7 weeks old, pathogen-free, antibody-devoid, and of hysterectomy origin(4). Swiss albino mice were weaned when 4 weeks of age and used in the experiments when they were 5 to 6 weeks of age.

Virus. Egg (26 passages) and mouse (232 passages) adapted Shope's (S-15) strain of swine influenza virus was used. Test virus was prepared by dilution of infective chick embryo allantoic fluid harvested 48 hours after inoculation; sealed in 1 ml ampules and stored at −60°C.

Preparation anti-S-15 conjugate. Antisera were obtained from pathogen-free pigs which had received 5 increasing inoculations of S-15 virus over a 3-month period. The pooled serum had a titer of 1:10,240 hemagglutination inhibition (HI) units per ml. Globulins were fractionated with ammonium sulfate (1.39 M), conjugated with crystalline fluorescein isothiocyanate† and dialyzed against saline solution (0.05 M) to remove free fluorescein (5).

Dialyzed conjugate was both absorbed with liver powder(2) and chromatographed in DEAE cellulose‡(6). Conjugate so treated with liver powder and chromatographed gave less nonspecific staining of granulocytes than conjugates that have been either chromatographed or absorbed with liver powder alone. Fluorescein-protein ratio was determined by absorption at 280 mμ and 495 mμ on a Beckman model DB spectrophotometer(6). A dye protein ratio of 2.5-5.4 $\times 10^{-3}$ was most suitable for staining. A dilution test and HI titration indicated that 160-320 HI units/ml was necessary for specific IF with this conjugate.

Sections and stains. Tissue was taken at necropsy, frozen in dry ice, and stored at −20°C. Cryostat sections (4 to 6 μ) were fixed in cold acetone (4°C) for 10 min; dried in an incubator (37°C); rinsed with phosphate buffered saline solution (0.01 M, pH 7); overlaid with conjugate without complement; and incubated in a moist chamber at 37°C for 40 minutes. Excess conjugate was washed out by placing the sections in 4 changes of buffered saline during a 30-minute period. A distilled water rinse removed the saline and the sections were mounted in phosphate-buffered glycerol. Specificity of staining under UV§ light was assured through positive and negative control tissues (infected and normal) mounted on each slide along with the test tissue.

Experimental procedure. One hundred and twenty mice were inoculated intranasally-orally (0.015 ml) with 100 mouse ID_{50} while under light ether anesthesia. Five mice were killed on 0, 4, 8, 12, 16, 20, and 24 hours, and thereafter every day for 10 days. Lungs and heads of these mice were sectioned to determine IF in bronchi, alveoli, or turbinates.

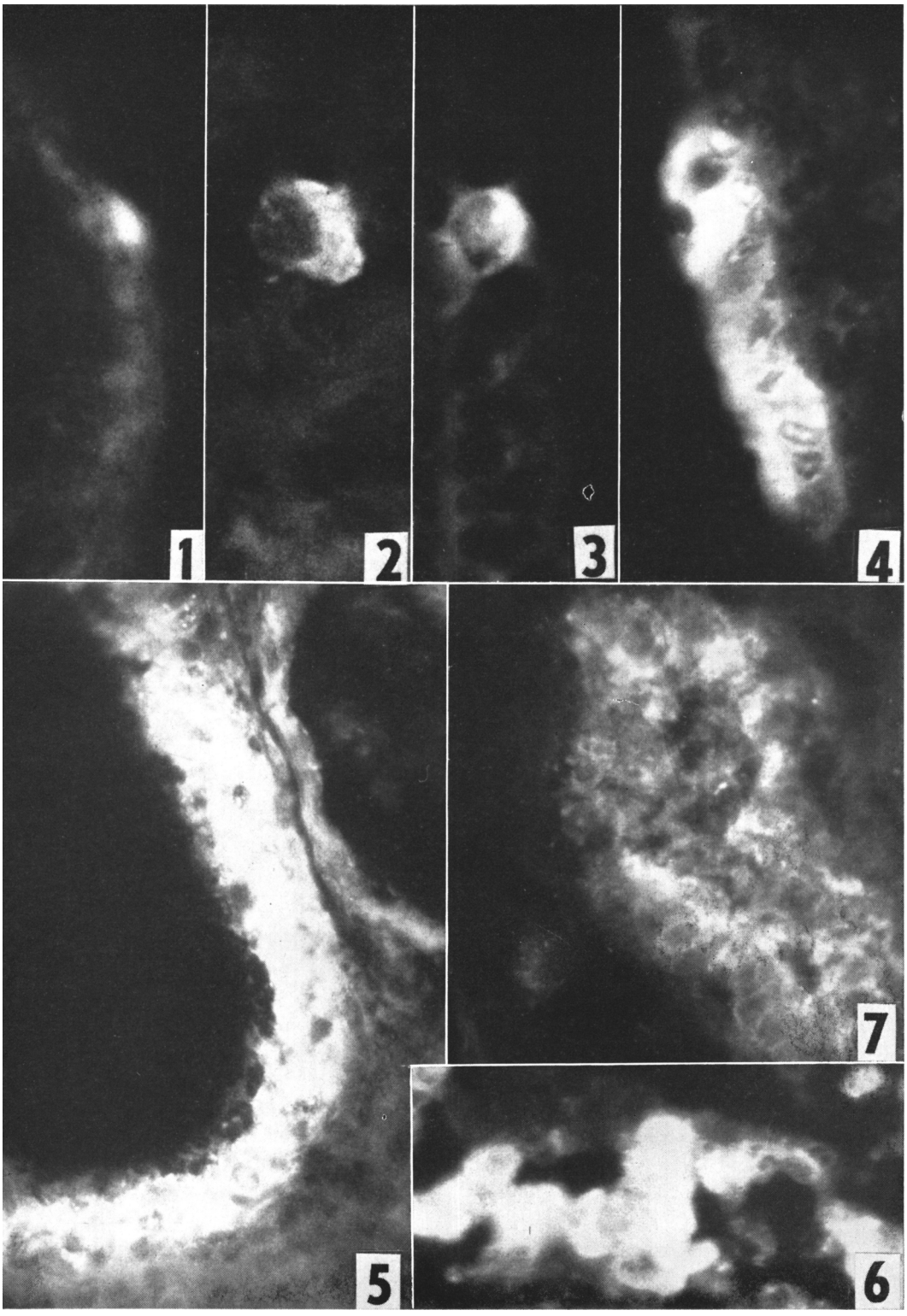
Results. Clinical signs appeared 3 days postinoculation; roughened haircoat, dyspnea, and anorexia. Lesions in lungs appeared 4 days postinoculation, became most intense on the 7th day, and completely disappeared by the 10th day (Fig. 13).

IF was not detected in the lungs or turbinates until 8 hours postinoculation at which time it was found within single cells in the bronchial epithelium; only 4 to 6 cells per section (Fig. 1). IF at this time was pri-

† Lot No. 1036, Sylvania Chemical Co., Orange, N. J.

‡ Type 20, Brown Co., Boston, Mass.

§ Leitz, Ortholux Microscope equipped with a dark-field condenser, HBO-200 L-2 light source, 2 mm UGI 'exciter' filter and Euphos barrier filter, E. Leitz, Inc., New York.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marily cytoplasmic (Fig. 2); nuclear fluorescence was observed rarely (Fig. 3).

At 16 hours, IF was predominantly located in bronchial epithelium in the apical lobes of lungs and a few discrete fluorescent cells (2 or 3 per section) were found in alveolar septum. The fluorescence was usually cytoplasmic; occasionally nuclear or both cytoplasmic and nuclear.

At 20 hours, IF was still primarily in the bronchial epithelium; however, the number of fluorescent bronchi were more numerous than they were at 16 hours. Also the number of fluorescent cells (cytoplasmic) and intensity of IF had increased. By this time, clusters of cells were fluorescing (Fig. 4) rather than single cells as at 8 and 16 hours. Only occasional fluorescent cells were seen in the alveoli.

At 24 hours IF was abundant in the bronchial epithelium. The apical and middle parts of the lung were reacting strongest at this time. The entire bronchial epithelium was often fluorescent in this region (Fig. 5) whereas only a few cells were fluorescent in the diaphragmatic lobe. Desquamated fluorescent cells were seen in the bronchial lumen (Fig. 6). IF occurred in the cytoplasm, nucleus, or in both; cytoplasmic IF was more conspicuous. At 24 hours still only a few fluorescent cells were seen in the terminal bronchioles or alveoli. The turbinate epithelium was negative.

At 48 hours IF occurred in alveolar septum and bronchial lumen. Desquamation of bronchioles was more prominent in the apical lobes (Fig. 7) than in the middle region of the lungs. Epithelia of the bronchi were often entirely fluorescent in the middle region, but IF was spotty and sparse in the apical lobes where much sloughing had occurred. IF was more conspicuous in the bronchial epithelium of the diaphragmatic lobes than at 24 hours. Fluorescent cells in the alveolar septa (Fig. 8 and 9) were more numerous than before. IF occurred in the turbinate epithelium of one mouse at 48 hours.

On the 3rd day IF occurred in the middle parts of lungs (bronchioles and alveoli), but not in the diaphragmatic part. In the alveoli IF was intense near the cell membranes (Fig. 10).

On the 4th day IF was seen in bronchioles in all parts of lungs; however, it had begun to diminish in the apical and middle regions. Many of the epithelial cells had disintegrated, releasing fluorescent substances into the lumen of the bronchioles. Others had sloughed and were present as fluorescent cells in the lumen. The infection was still spreading as indicated by the presence of new IF foci. Alveolar IF occurred in all parts of the lung; however, it was confined to specific areas. In some regions IF was more pronounced in the alveoli adjacent to fluorescent bronchioles. In some bronchi elaborated fluorescent material was found adjacent to intact fluorescent cells. In these cells, IF was intense in the region bordering the lumen. IF occurred in the epithelium of nasal turbinates in one mouse on the 4th day (Fig. 12).

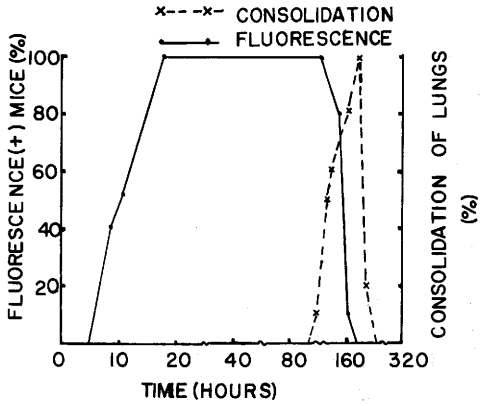

FIG. 13. Time of appearance of fluorescence and consolidation in mice lungs following inoculation with S-15 virus.

On the 5th day, fluorescent exudate was extensive in bronchial lumen and many epithelial cells had sloughed. The number of

FIG. 1-7. Progress of influenza (S-15) infection in bronchi of experimentally infected mice. 1. Outer edge of a cell fluorescing, 8 hr postinoculation (1000×). 2. Single cell fluorescing (cytoplasmic), 8 hr (1000×). 3. Single cell fluorescing (cytoplasmic and nuclear), 8 hr (1000×). 4. Cluster of cells fluorescing (cytoplasmic and nuclear), 20 hr (500×). 5. Complete epithelial lining of a bronchus fluorescing, 24 hr (500×). 6. Desquamation of fluorescent cells from bronchial epithelium, 24 hr (500×). 7. Fluorescing exudate in a bronchial lumen, 48 hr (500×).

fluorescent cells had decreased in the bronchial mucosa. Fluorescent macrophages were seen in the alveoli. IF occurred in all parts of the lungs. Both nuclear and cytoplasmic fluorescence occurred. No IF was seen in epithelium of the turbinates.

On the 6th day, IF was rare in bronchioles of the apical region but a few fluorescent cells were still present in the middle and the diaphragmatic regions. IF occurred in only a few alveoli.

On the 7th day, a few mononuclear cells with fluorescent nucleus and cytoplasm were found indiscriminately scattered throughout

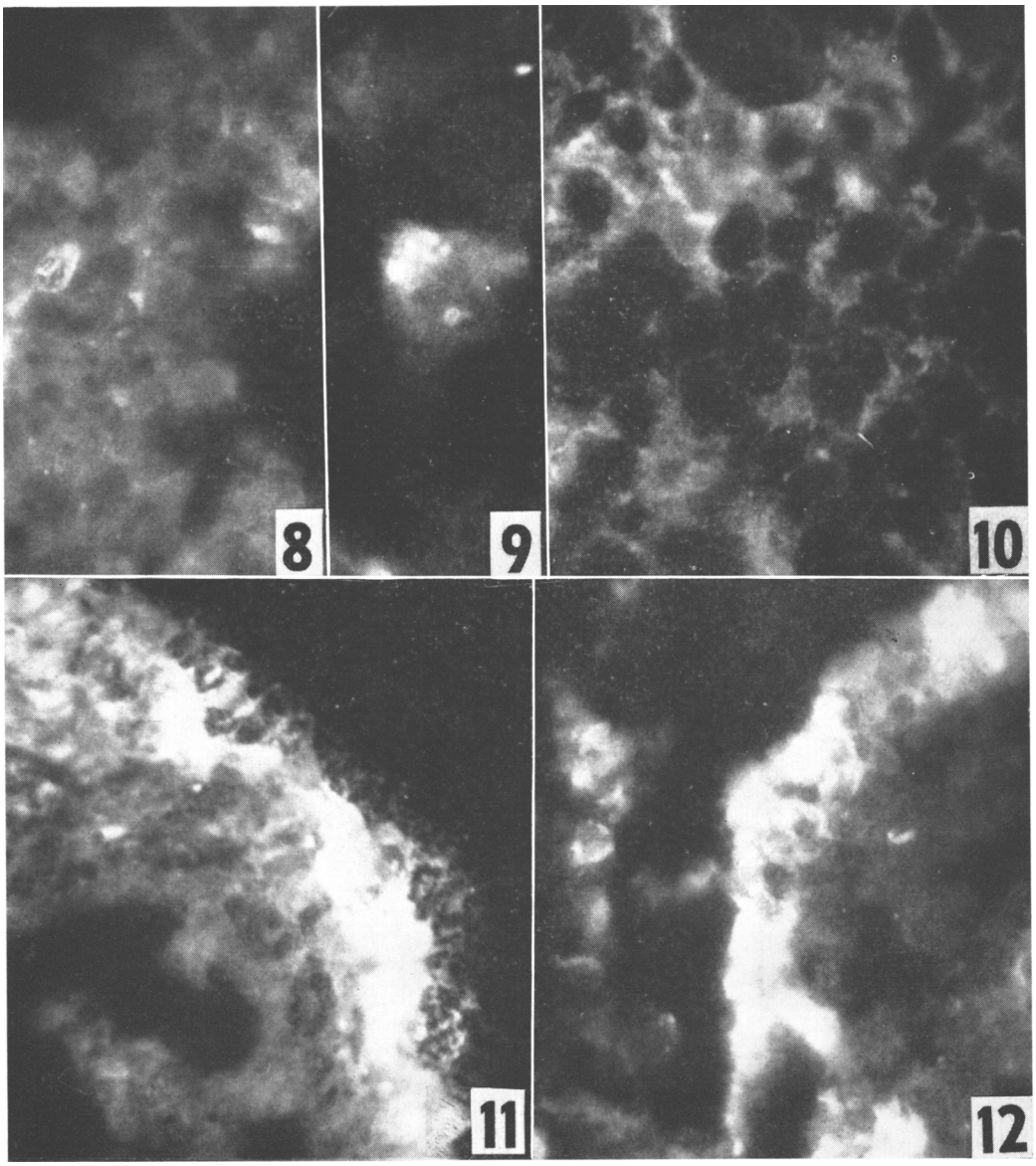

FIG. 8-12. Immunofluorescence showing in mice infected with influenza. 8. Fluorescent cells in alveolar septa, 48 hr cytoplasmic and reticular fluorescence of nucleus (500×). 9. Cytoplasmic and only nucleolar fluorescence (1000×). 10. Fluorescence of cytoplasmic membrane, while the nucleus and adjacent area nonfluorescent, 3 days (800×). 11. Fine threads of fluorescing material coming out of bronchial cells, 4 days (500×). 12. Fluorescence in turbinate mucosa, 4 days (500×).

the lung parenchyma. On days 8, 9, and 10, no IF was found in the lungs or turbinates.

Discussion. The initial site of virus infection appeared to be in the bronchial epithelium; IF was not usually seen in alveoli until 24 hours postinoculation. Large and small bronchi reacted with the same intensity. It appeared that the spread of viral infection took place as follows: Virus invaded the bronchial epithelium and spread to surrounding cells by contact. Infected cells sloughed, disintegrated, and released virus particles which invaded adjacent cells. The infection appeared to proceed from bronchi to bronchioles to alveoli. Fluorescent macrophages, probably from phagocytizing the virus-infected debris and cells, were found in bronchial epithelia and alveolar septa during all stages of the infection. The role of macrophages in the spread of viral infection could not be specifically determined.

Hers(3) concluded, "virus pneumonia in mice can begin in alveoli instead of spreading down by bronchial tree." However, from our observation invasion of bronchial epithelium was the initial step in infection; the alveoli became infected later. At 16 and 24 hours when bronchial fluorescence was numerous only a few discrete cells could be detected in terminal bronchioles and alveoli. The discrepancy between the observation of Hers and ours could be due to the fact that Hers used overwhelming virus inoculation (0.05 ml of undiluted amniotic fluid) intranasally carrying virus directly into the alveoli. Research by Watson(8) in another test system supports our observations. Watson(8) used a higher multiplicity of virus and detected nuclear fluorescence in amniotic cells of chick embryo 1 hour following inoculation. He observed that the time required for initial appearance IF reaction decreased as the amount of inoculum was increased.

In the early stages of infection cellular IF was usually cytoplasmic and occasionally nuclear. In the cytoplasm IF was concentrated toward the lumen border of the bronchial epithelium, whereas in the alveoli IF congregated along the cell membrane giving a network appearance to the preparation (Fig. 10). In some nuclei the entire chromatin network was fluorescent while in others only the nucleolus fluoresced (Fig. 8 and 9). In this study IF was first observed 8 hours postinoculation. In monkey kidney cell culture infected with influenza A strain soluble antigen was detected in the nucleus by IF technique as early as 4 hours after infection; antigen concentration reached maximum 12 hours after infection(7). Hers(1) found cytoplasmic IF in the bronchial epithelium of mice lungs 1 hour and in alveolar cells 2 hours following massive inoculation of influenza virus by nasal route. It is not clear how virus is transmitted from one cell to another in the early stages of infection. At 8 and 16 hours, only a few discrete fluorescent cells could be found (4-6 per section). By 20 to 24 hours, clusters of fluorescent cells were found. There had been no apparent disintegration of any of the infected cells up to this time. Similar observations have been recorded by Sugiura(7) in tissue culture and Watson (8) in embryonated eggs. In many of the fluorescent cells of the bronchial epithelium threads of fluorescent material extended into the lumen (Fig. 11). Sugiura(7) observed similar structures and considered them to be virus particles (V antigen) emerging from the cells. Morgan *et al*(9) observed similar cytoplasmic protrusion in infected cells containing virus particles in the process of emergence. Using ferritin conjugated antibody they further demonstrated that many of these cytoplasmic processes are antigenically similar to virus and constitute probably what is known as incomplete virus. IF was found in the turbinates of only two mice 2 and 4 days after virus inoculation (Fig. 12). Liu(2) found fluorescence in ciliated cells of turbinates of ferrets infected with mouse adapted Lee virus on the second day after infection but none with egg adapted Lee strain. Dublin(10), and Hers(3) did not record any change in turbinates in mice infected with S-15 virus.

Summary. A specific immunofluorescent (IF) technique was developed for swine influenza virus using fluorescein conjugated immune globulins. IF technique was employed to study the progressive pathogenesis of S-15 virus in mice. IF was detected in bronchial

epithelium 8 hours after inoculation. Fluorescence, at this stage, was mostly cytoplasmic, however both cytoplasmic and nuclear fluorescence occurred in a few cells. IF reached a peak on the second and third day in the bronchi and usually spread to alveoli by the second day post inoculation. After the fourth day post inoculation, IF diminished in the alveoli and bronchi and disappeared after the 7th day. IF indicated that both cytoplasm and nuclei of infected cells are involved in the synthesis of viral antigens. IF was limited to macrophages and epithelial cells of bronchi, alveoli, and turbinates. It was concluded that influenza virus progressively infects bronchi, bronchioles, alveolar ducts, and alveoli during the course of infection.

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Induction of Tolerance to Skin Homografts by Administering Splenic Cells to Pyridoxine-Deficient Mice.* (29202)

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Induction of tolerance to tissue homografts can be achieved in adult mice through parabiotic union or by suitable administration of appropriate viable splenic cells or cellular extracts. The extensive literature pertinent to this subject has been reviewed recently(1-3). Utilization of cellular extracts for production of tolerance possesses distinct advantages(1) and we have, accordingly, undertaken a series of investigations designed to ascertain the effectiveness of various cellular components derived from splenic cells syngeneic with those of the skin donor. In these experiments, the cellular components are administered to recipients in a temporary state of pyridoxine deficiency. Rationale for this experimental procedure derives from numerous demonstrations of immunological unresponsiveness produced by this deficiency(4). Since the utility of intact cells in induction of tolerance is well-documented, we considered it advisable to ascertain the value of such viable cells under our experimental conditions before embarking upon a prolonged study involving use of cellular components. The present paper records results of these initial experiments which demonstrate successful achievement of tolerance in mature mice which had received splenic cells while in a prior state of pyridoxine deficiency.

Materials and methods. Animals and diets. Male mice of the CBA/J, C3H/HeJ and A/HeJ strains, 4-5 weeks of age, were obtained from the Roscoe B. Jackson Memorial Laboratory. Animals of the recipient CBA/J strain received upon arrival the purified control (complete) diet described in Table I. The pyridoxine-deficient diet fed subsequent-

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