Immunofluorescent Study of B4 Coxsackievirus Valvulitis in Mice.* (32037)

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Recent reports from this laboratory have demonstrated the importance of coxsackievirus B4 as an etiologic agent in the production of experimental valvulitis in laboratory animals(1,2,3). Furthermore, in a previous report it was postulated that some instances of acute and chronic valvulitis in man may be viral in origin(4). The identification and localization of coxsackievirus antigen in the valve of human hearts would provide further substantiation of this postulate. Such investigations are presently underway in this laboratory.

In the present study an experimental model of both acute and chronic viral valvulitis was studied by the direct immunofluorescent antibody technique in order to correlate the histopathology of this disease with the distribution of the coxsackievirus antigen in chronically diseased valves and mural endocardium.

Materials and methods. Virus stock. The coxsackievirus used in this experiment was originally recovered by Kibrick and Benirschke from a 10-day-old infant who died of encephalohepatomyocarditis(5). The virus was obtained as monkey kidney culture passage strain. Virus for this experiment was prepared in rhesus monkey kidney cultures according to technique previously described (6). Control fluid from monkey kidney culture free of virus was also obtained. Virus and control fluid were stored at −65°C.

Mice. A random breed strain of HaM/ICR mice was used in this experiment.

Inoculation of the virus and collection of tissues. Thirty young adult mice (18-20 g) were inoculated intraperitoneally with 0.1 ml of fluid containing either 10^3 50% tissue culture infective dose ($TCID_{50}$) or 10^5 $TCID_{50}$. Six infected mice were sacrificed separately at intervals of 3 days, 1, 2, 4, and 7 weeks and their hearts were excised for examination. Control animals injected with 0.1 ml of virus-free fluid from monkey kidney culture were sacrificed and their hearts processed simultaneously.

Antiserum and staining. Fresh frozen sections were stained with fluorescein labeled rabbit antiserum. Antiserum was prepared in rabbits by two intravenous injections of 10 ml of coxsackievirus B4 suspension at 2-week intervals. Globulin fractions were separated by treatment of whole serum with ammonium sulfate. Fluorescein isothiocyanate was conjugated with the globulin fraction and the direct immunofluorescent technique employed (7). A Reichert microscope with appropriate ultraviolet light source and filters was used for photomicrography. After fluorescent microscopic examination the sections were stained with hematoxylin and eosin for histopathologic study.

Specificity of fluorescent staining. The specificity of the staining reaction was indicated by the following: (1) tissue from control animals displayed no fluorescence, (2) the staining reaction was greatly weakened or absent when the sections of infected heart were treated with unconjugated antiserum, followed by application of conjugated antiserum, and (3) infected heart tissue showed no fluorescence after treatment with fluorescein-conjugated, normal rabbit serum.

Results. At least 3 or 4 sections from each infected heart were examined. Two or three valves were visualized microscopically in most hearts. Coxsackievirus antigen was detected in the valves and mural endocardium as well as in the myocardium and pericardium by the appearance of specific intracytoplasmic fluorescence. The amount of antigen detectable as fine, bright, yellow granules varied during the different periods of infection.

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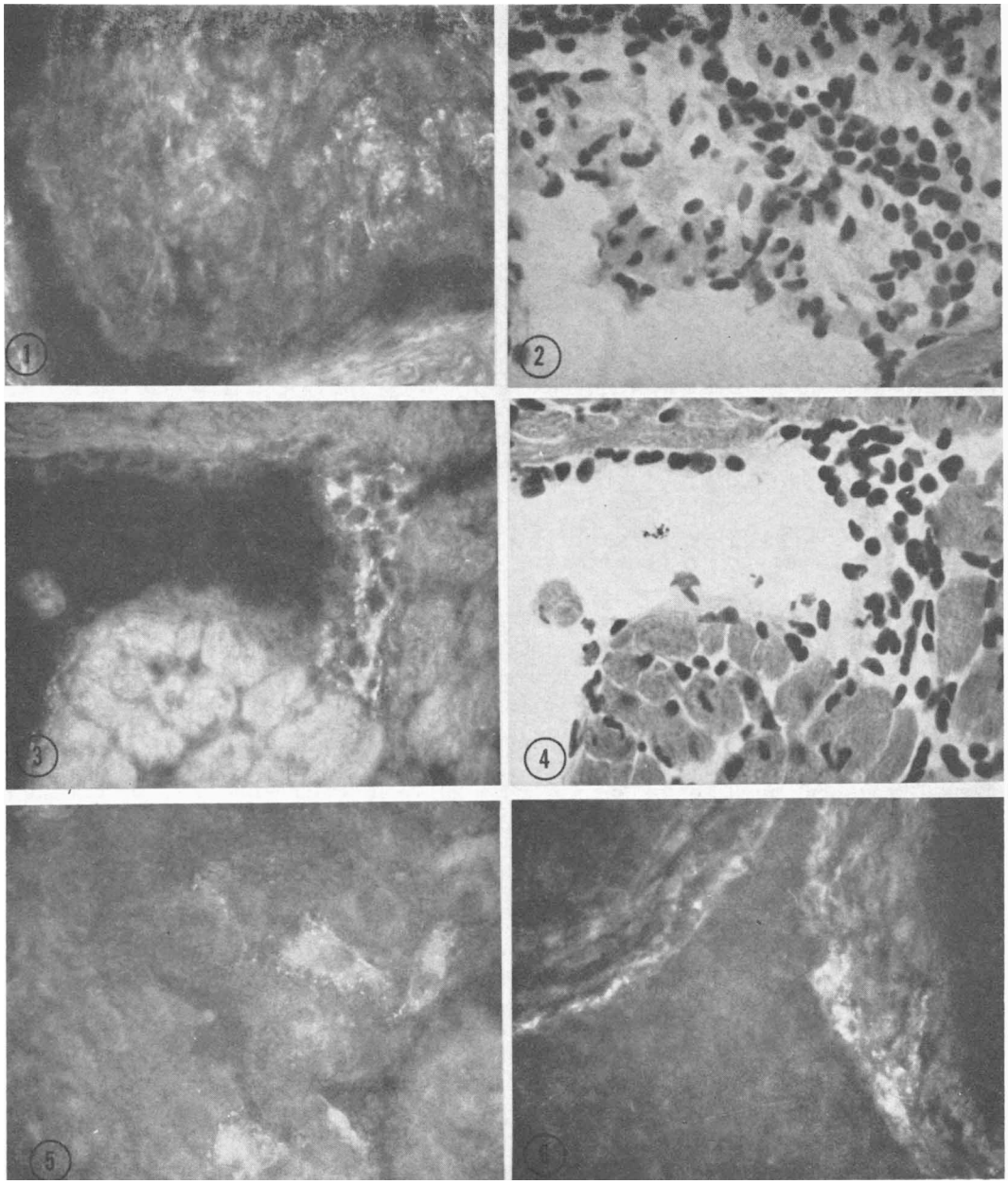

FIG. 1. A tricuspid valve on 3rd day after inoculation, showing granular cytoplasmic fluorescence in connective tissue cells. Direct fluorescent antibody stain, DFA stain, × 480.

FIG. 2. The same tricuspid valve showing edema and round cell infiltration in connective stroma. Apparently, most of the antigen-bearing cells are mononuclear cells. Hematoxylin and eosin stain, H & E stain, × 480.

FIG. 3. Mural endocardium of right ventricular wall on 3rd day after inoculation showing granular cytoplasmic fluorescence in the lining endothelial cells and infiltrating mononuclear cells. DFA stain, × 480.

FIG. 4 The same area of mural endocardium as Fig. 3. showing endothelial cell proliferation and mononuclear cell infiltration. H & E stain, × 480.

FIG. 5. A tricuspid valve 1 week post infection showing fine, granular fluorescence in the cytoplasm of endothelial cells. WFA stain, × 780.

FIG. 6. A pulmonary valve after 1 week infection showing fluorescence in the lining endothelial cells. DFA stain, × 480.

Specific fluorescence was first demonstrated in the tricuspid valves and mural endocardium of the right ventricle on the third day after inoculation. Involvement of the myocardium and pericardium were quite advanced by this time indicating even earlier involvement. In the affected valves there was a widespread involvement of the stromal cells which showed a fairly intense granular cytoplasmic fluorescence (Fig. 1). Fluorescence was absent from the valvular endothelium at this time. Hematoxylin and eosin staining showed that the antigen-bearing cells within the valves were mostly round or spindle-shaped cells with deeply basophilic nuclei (Fig. 2). These cells were thought to represent inflammatory and proliferating fibroblastic components.

Fluorescent microscopy of the mural endocardium showed marked involvement of the endothelial cells with virus antigen. This was especially prominent in the right side of the heart. At the junction of the trabecular carneae with the right ventricular wall, there often appeared clusters of bright fluorescent staining cells which by hematoxylin and eosin were identified as proliferating endothelial and fibroblastic cells (Fig. 3, 4).

Bright patches of scattered fluorescence were noted in the tricuspid and pulmonary valves after one week of infection. The endothelial cells of the leaflets were noted to bear antigen in the form of bright finely granular aggregates within their cytoplasm (Fig. 5, 6). High concentrations of antigen were found at the base of the tricuspid valves (Fig. 7). Weak fluorescence was occasionally noted in the mitral and aortic valves. The involvement of mural endocardium was usually widespread at this time. Histologically, mononuclear cell infiltration was prominent at the base of the tricuspid valve (Fig. 8). The valvular leaflets themselves showed moderate edema and cell proliferation.

By the second week post infection, the antigen could be located in the aortic and mitral valves. Valvular endothelial cell involvement was quite prominent by this time. Antigen was distributed either at the surface endothelium or in the deep stroma of the valves (Fig. 9, 10). The hematoxylin and eosin staining showed that the cellular types in the fluorescent areas were proliferating endothelial cells, fibroblasts and infiltrating mononuclear cells (Fig. 11, 12). Mural endocardial involvement was quite marked and myocardial necrosis extended to the subendocardial layers.

By the fourth week post infection the concentration of antigen in the affected valves seemed to be less, but localization of the antigen was much more discrete. The majority of the fluorescence was located distinctly within a few swollen endothelial cells (Fig. 13). The histological features revealed by hematoxylin and eosin staining of the same section displayed an active cellular proliferation (Fig. 14). There were also considerable scarring and cellular proliferation in the mural endocardium. (Fig. 15).

The distribution and localization of the antigen remained unchanged at the seventh post infection week. In many instances the chordae tendineae and attached papillary muscle of the mitral valve showed high concentrations of viral antigen and marked degrees of tissue reaction (Fig. 16).

Discussion. The present report provides additional information regarding the pathogenesis of coxsackievirus B4 valvulitis and mural endocarditis. The evidence clearly indicates that involvement of the valves and mural endocardium can occur concurrently with coxsackievirus B4 myocarditis. The valvular involvement occurs as early as the third post infection day and is manifested primarily by an invasion of the stromal cells of the valvular leaflets by the virus. The area of most intense fluorescent reactions is consistently located at the base of the affected valves. It is possible that valvular involvement is mediated by a direct extension from the adjacent myocardial lesion. The involvement of the valves and mural endocardium of right side of the hearts is more frequent, especially in the early stage of infection. This is probably because the intraperitoneal method was used to produce infection. It is interesting to speculate as to whether a higher incidence of left sided valvular lesions would be encountered if another route of infection was employed, *e.g.*, transbronchial. Endothelial cells on the valvular leaflets seem to be in-

vaded only after the first or second week of infection at a time when the stromal reaction is greatest. It is particularly interesting that as the valvular lesion progresses to fourth and

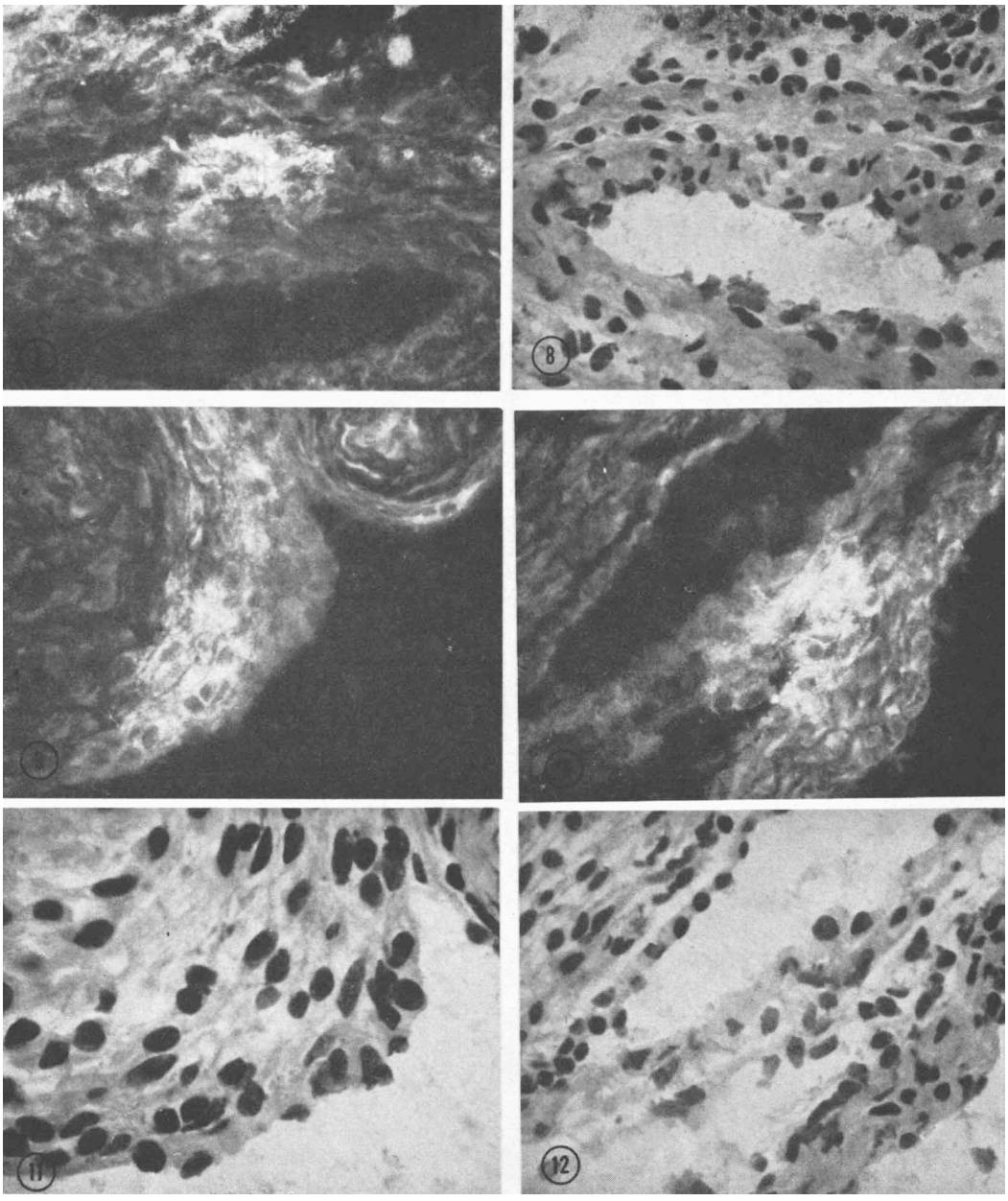

FIG. 7. A tricuspid valve 1 week post infection showing bright, cytoplasmic flouresecence at base af valve. DFA stain, × 480.

FIG. 8. The same valve showing fibroblastic response and mononuclear cell infiltration corresponding to the site of the bright fluorescence. H & E stain, × 480.

FIG. 9. An aortic valve on 2nd week post infection showing bright, cytoplasmic fluorescence in proliferating endothelial cells. DFA stain, × 780.

FIG. 10. A mitral valve on 2nd week post infection showing bright, cytoplasmic fluorescence in the connective tissue cells of leaflet. DFA stain, × 480.

FIG. 11. & 12. The same aortic (× 780) and mitral (× 480) valves showing endothelial cell proliferation and swelling. H & E stain.

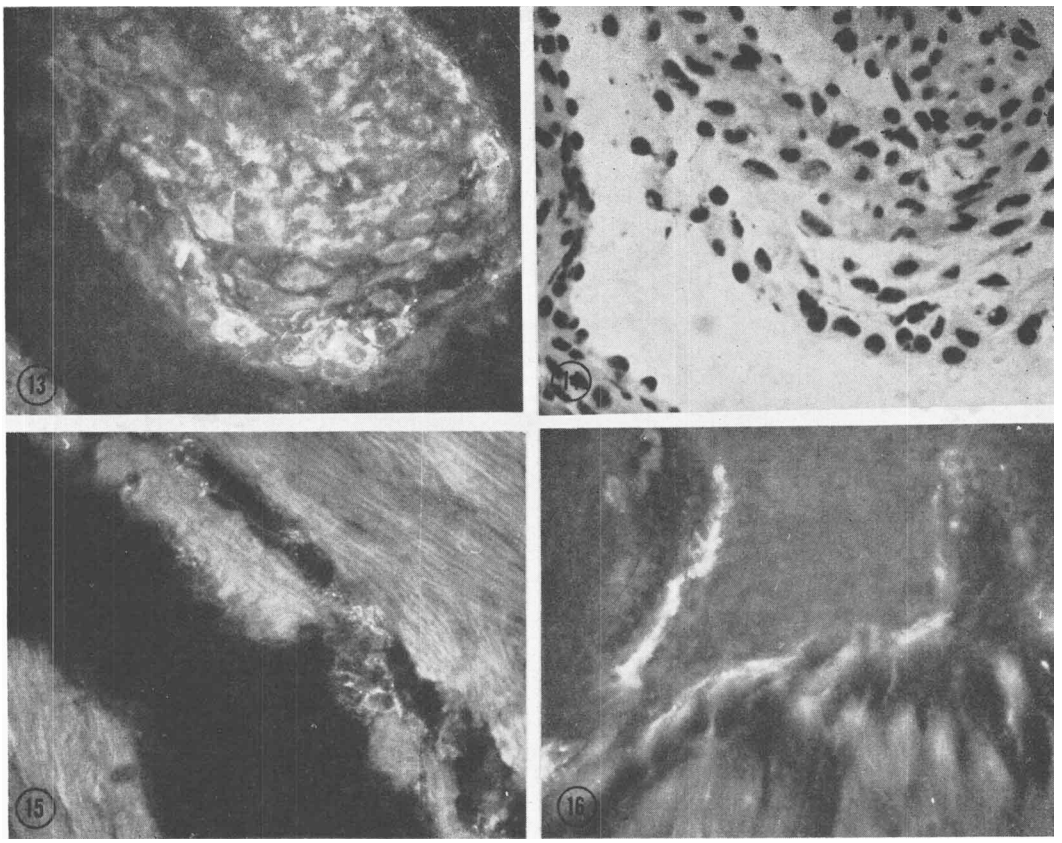

FIG. 13. An aortic valve 4th week post infection showing well localized, fluorescent antigen in cytoplasm of swollen endothelial cells. DFA stain, × 480.

FIG. 14. The same aortic valve showing swollen endothelial cells, stromal edema and fibrocytic proliferation. H & E stain, × 480.

FIG. 15. A portion of mural endocardium of left ventricle 4th post infection week, showing fluorescent granules in proliferating bands. DFA stain × 480.

FIG. 16. A papillary muscle of left ventricle at 7th post infection week, showing bright fluorescence and marked tissue reaction in chordae tendineae. DFA stain, × 480.

seventh weeks, the concentration of antigen and the extent of its distribution in the affected valves becomes significantly reduced. It is at this time however that there is a much more discrete localization of the antigen to the endothelial cells. The antigen is persistently present even in the fibrotic chordae tendineae as well as the scarred mural endocardium. It is evident that the coxsackievirus B4 valvulitis and mural endocarditis may become chronic while the virus or a viral related antigen still remains *in situ*.

Summary. An experimental model of both acute and chronic viral valvulitis was studied by direct immunofluorescent antibody technique with parallel histopathologic observation. Coxsackie B4 virus antigen was visualized in the affected valves and mural endocardium of acute infections and remained *in situ* even after the tissue developed scarring. The antigen was restricted to the stroma of the infected valves in the early stages and became more discretely localized in the endothelial cells at a later period. The intense, specific fluorescent reaction at the base of the affected valves provided evidence of valvular involvement by direct extension from adjacent myocardial lesions. Involvement of valves and mural endocardium of right side of the hearts was more frequent, especially in the early stages of infection. The studies show that the coxsackievirus B4 can produce valvulitis in mice as well as pericarditis, myocarditis and endocarditis.

1. Burch, G. E., DePasquale, N. P., Sun, S. C., Mogabgab, W. J., Hale, A. R., Science, 1966, v151, 447.

2. Burch, G. E., DePasquale, N. P., Sun, S. C., Hale, A. R., Mogabgab, W. J., JAMA, 1966, v196, 349.

3. DePasquale, N. P., Burch, G. E., Sun, S. C., Hale, A. R., Mogabgab, W. J., Am. Heart J., 1966, v71, 678.

4. Burch, G. E., DePasquale, N. P., ibid., 1964, v67, 721.

5. Kibrick, S., Bernirchke, K., Pediatrics, 1958, v22, 857.

6. Mogabgab, W. J., Ann. Intern. Med., 1963, v59, 306.

7. Cherry, W. B., Goldman, M., Carski, T. R., Moody, M. D., USPHS Publ. 729, 1960.

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Rubella Hemagglutinin Prepared with Alkaline Extraction of Virus Grown in Suspension Culture of BHK-21 Cells. (32038)

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The preparation of a new type of alkaline extracted potent complement fixing (CF) antigen without serum components(3) suggested the possibility that this antigen might contain hemagglutinin activity. The report of Meyer(4) of agglutination of erythrocytes from one-day-old chicks by a rubella antigen prompted us to test our antigen with the same type of cell.

The present report describes the specific hemagglutination (HA) of young chicken erythrocytes with alkaline extracted rubella antigens prepared from infected BHK-21/13S cells grown in suspension culture. Factors which enhance both the preparation and testing of the antigen and the performance of the hemagglutination inhibition (HI) test are also indicated.

Materials and methods. Virus strain and cell culture techniques have been described previously(3). Aliquots of the seed virus (8th passage level in this laboratory) were stored at −70°C. The titer of this virus was about 10^7 $TCID_{50}$/ml. Fifteen ml of seed virus was inoculated into 800 ml of suspension culture with about 10^6 cells/ml. The infected cells were harvested on the 7th day.

In the HA and HI test, antigen and serum diluent was 0.4% bovine-albumin-borate-saline solution and erythrocyte diluents were 0.15 M NaCl-phosphate buffers with indicated pH(7). The blood of chickens at indicated ages was drawn from the heart using a 5 ml syringe containing 3 ml of Alsever's solution(6). From each of 10 chickens 0.5 ml to 1.5 ml of blood was collected, pooled and stored in Alsever's solution. A 10% stock suspension was prepared in Alsever's solution and stored up to a week at +4°C. The 0.25% working suspension was prepared daily in erythrocyte diluent. Serum specimens for HI tests were treated with kaolin and chicken red cells according to the arbovirus technique (7) with minor modifications: the specimens were diluted 1:5 with borate saline, pH 9.0 (0.2 ml of serum and 0.8 ml of borate saline), in a 15 ml conical centrifuge tube. An equal volume (1.0 ml) of 25% kaolin suspension (prepared in borate saline, pH 9.0) was added; the tubes were shaken vigorously 2 to 3 times during 20 minutes incubation at room temperature and centrifuged in the same temperature at 2,500 RPM for 30 minutes (International Centrifuge, Model PR-2, Rotor No. 269†). Without collecting the supernate,

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† Use of trade names is for identification only and does not constitute endorsement by the Public Health Service of the U. S. Dept. of HEW.