Precipitinogens of Rubella Virus-Infected Cells* (33486)

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Rubella virus and serum reactants, particularly concentrates of medium from infected cultures of the PS (porcine stable) line of pig kidney cells (1) and sera of convalescent patients or hyperimmunized baboons, revealed 2 distinct precipitating systems by immunodiffusion, which form the subject of this report. The antigens, and the corresponding precipitate bands, are called *theta* and *iota*.

Materials and Methods. Two strains of rubella virus were used: the M33-HPV77 vaccine strain (2); and the Mag strain, isolated in this laboratory (3). Infected PS cell cultures were maintained indefinitely at 34–35°, with medium changes every 7–10 days. The medium was Eagle's minimum essential medium, with 4 m*M* glutamine, 5% fetal calf serum, penicillin, and streptomycin. By the third week after inoculation, a state of "chronic" infection had been established, characterized by altered cell morphology and growth, and a fluctuating balance between cell destruction and proliferation, with continuing production of infective virus. Concentrations of 10^5–10^7 focus-forming units/ml were present in weekly harvests. Medium harvested from these PS-RV carrier cultures was stored at —20°, later pooled and concentrated ~100× by dialysis against Carbowax (polyethylene glycol 20,000, Fisher Scientific Co.).

The resulting antigen reactants were examined by immunodiffusion. Tests were done on 75 × 25-mm glass slides covered with 2.4 ml of gel, consisting of 0.45% agarose in 0.1 *M* Tris buffer, pH 8.0, 0.1 *M* NaCl, and 15 m*M* sodium azide. The standard 3-mm diameter cup held ~10 μl of reactant. Edge-to-edge distance between antigen and antibody cups was usually 4 mm. Slides were kept in moist boxes at ~20°. Precipitate bands were first seen at 8–12 hr. Tests were read at 18 and 42 hr.

The antigen reactants were standardized against one serum from a hyperimmunized baboon, as previously described for poliovirus (4). The antiserum used as standard came from a baboon infected intranasally with rubella virus (Mag strain), and reinoculated with Mag, by i.m., i.p., or i.v. routes, at 5-, 6-, and 20-month intervals; the serum was taken 2 weeks after the last inoculation. The position and character of precipitate bands formed by varying dilutions of antiserum and antigen reactant were determined, and the equivalence zone for each antigen–antibody system was defined. The highest dilution of standard antiserum giving distinct precipitation in the equivalence zone with any dilution of a specific antigen reactant was taken as containing 1 unit of homologous precipitin/μl; and that particular dilution of the antigen reactant was held to contain 1 unit/μl of the precipitinogen. The diluent was 0.15 *M* NaCl.

Human serum precipitin was assayed, and its identity was checked, by comparison of the position of its precipitate with that formed by the standard baboon antiserum with the same dilution of antigen reactant (5). Serum specimens and antigen concentrates were stored at —20°.

Rubella hemagglutinin-inhibiting (HAI) antibody was estimated by a method (6) modified from that of Stewart *et al.* (7). Sera were treated with heparin–$MnCl_2$, instead of kaolin, to remove nonspecific inhibitors (8). The HAI titer of a serum was expressed as the reciprocal of the highest dilution which completely inhibited eight 50%-hemagglutinating doses (HAD_{50}) of virus.

Results. Immune precipitation with rubella virus reactants. The θ and ι bands formed by the standard antiserum with 4 different HPV77 and Mag concentrates are shown in Fig. 1. In tests with varying dilutions of antiserum, the position of the equivalence zone for both precipitating systems was at ~0.6 of the distance from antigen to antibody

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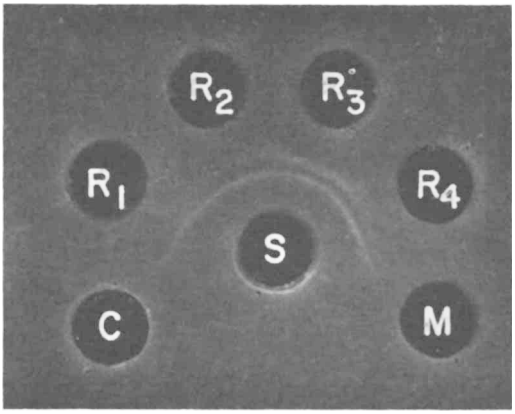

FIG. 1. Formation of θ and ι precipitates by rubella reactants diffusing in agarose. S = standard baboon rubella antiserum, absorbed with preparation C. Peripheral cups contain various antigen reactants. Except for R_1, these consist of 100× concentrates of medium from the following PS cultures: C, uninoculated; M, infected with measles virus; R_2, infected with rubella virus, HPV77 strain; R_3 and R_4, infected with rubella virus, Mag strain (2 different preparations); R_1 contains an alkaline extract of BHK21/WI-2 cells infected with rubella virus (Mag). The 2 bands of precipitate are best seen with preparation R_3. The ι band is the one that is further from the antigen cup.

cup. No precipitate was seen, with a zone of equivalence closer to the antigen cup, which might have been related to aggregation of whole rubella virions. The θ and ι precipitinogens appear to be single determinants, and no qualitative differences have been found between those generated during infection with HPV 77 virus and the corresponding antigens induced by Mag. The separate identity of θ and ι was shown by absorption tests. Some human sera, as will be seen, possessed only anti-θ antibody. When mixed in optimal proportions with a PS-RV concentrate, they abolished its θ reactivity, but spared its ι.

Specificity of the reactions. The several baboon antisera all had antibodies against one or more bovine serum constituents, and were therefore preabsorbed with control uninoculated PS culture concentrate. This removed no anti-θ or anti-ι antibody, but eliminated all reactivity against the control preparation. The specificity of the reactions was confirmed with concentrates made from PS cultures showing characteristic degeneration after infection with measles (Schwarz strain), dengue 1 (Hawaii), dengue 2 (TH-36), or respiratory syncytial virus (a recent isolate from this laboratory). The rubella antisera formed no θ or ι bands with these reactants; nor was either antibody absorbed by them. The dengue virus preparations gave type-specific precipitates with their homologous mouse antisera.

Formation of θ and ι in cells other than PS-RV. Synthesis of θ and ι, while stimulated only by rubella virus among those agents tested, was not restricted to the chronically infected PS-RV cultures. Both antigens were present in "acutely" infected PS cultures 5 days after exposure to Mag virus at a multiplicity of ~1, though only at 1/8 to 1/4 of the levels usual for PS-RV. Two other cell lines were tested. Concentrates from infected BHK21/WI-2 (9) and LLC-MK2 cultured (10) contained both θ and ι. With the BHK line, the actual cells were a relatively rich source, yielding moderate amounts of both antigens (Fig. 1) after extraction with 0.1 *M* glycine, pH 9, at 36° for 36 hr (11). With PS-RV, by contrast, the antigens were present in high concentration in the medium, yet no θ or ι activity was noted in an homogenized, 3× frozen-and-thawed 20% saline suspension of the packed cells. Only a trace of ι appeared after alkaline extraction; with no increase on treatment with EDTA at pH 7 (1 part of 0.3 *M* EDTA to 2 parts of cell extract, consisting of packed cells taken up in 3 vol of 0.1 *M* glycine, pH 9). Levels of ι and θ antigen in PS-RV culture-fluid concentrates were not affected by treatment with glycine or EDTA, or by removal of floating cells and cell debris from freshly harvested medium prior to storage at −20°.

Size of the antigenic particles. The position of the θ and ι equivalence zones places each antigen on particles small enough to diffuse readily in 0.45% agarose, and clearly much smaller than the rubella virion. In keeping with this, neither antigen was sedimented from unconcentrated medium spun at 10^5 g for 3 hr, a force sufficient to sediment intact virus. Other studies indicate sedimentation coefficients for both antigens of between 4S and 7S (12).

Relationship of θ and ι to rubella virus chick-cell hemagglutinin (HA). Aliquots of a PS-RV concentrate were absorbed with packed red cells from 1-day-old chicks or sheep. This concentrate had been made from PS-RV cultures maintained in medium containing calf serum treated with kaolin to remove any nonspecific inhibitors of rubella HA, which might have interfered with the adsorption of θ or ι to chick red cells. Complete removal of chick-cell HA did not affect its θ or ι content; nor did treatment with sheep red cells. Other findings, too, spoke against identification of ι or θ with rubella virus HA. Vero-cell extracts of high HA titer (13) contained only traces of the 2 antigens. In contrast, even the best precipitating reactants derived from PS-RV cultures had little or no specific rubella HA activity (HAD_{50} dilution $\leq$1 in 8).

Anti-θ and anti-ι reactions of human sera. Although few human sera have been tested, it is already clear that patients with rubella develop both anti-θ and anti-ι precipitins. Sera had been collected during the first 3 months after onset of illness, and again 5 years later. In general, anti-θ precipitin was detected earlier than anti-ι, *i.e.*, within 1–3 days of the appearance of the rash, as opposed to about 7 days for anti-ι. Anti-θ antibody rose to higher levels than anti-ι, and apparently persisted with little or no decline in titer for at least 5 years; whereas anti-ι precipitin increased more slowly, and fell away sooner, being barely detectable, if at all, 5 years after the acute illness. Acute- and convalescent-phase sera from a child with rubella were fractionated by zonal sedimentation in preformed linear sucrose gradients, and tested for sensitivity to 2-mercaptoethanol (2-ME). Procedures and criteria were those used by Schluederberg (14), but 0.05 *M* 2-ME was substituted for 0.1 *M*.The earliest detectable anti-θ and anti-ι precipitins were mostly 2-ME-sensitive, with sedimentation coefficients in the region of 19S. They were presumably IgM molecules. In later sera, both antibodies were found in the fractions containing IgG, and were resistant to 2-ME. Figure 2 compares the development of HAI, anti-θ and anti-ι antibodies in 3

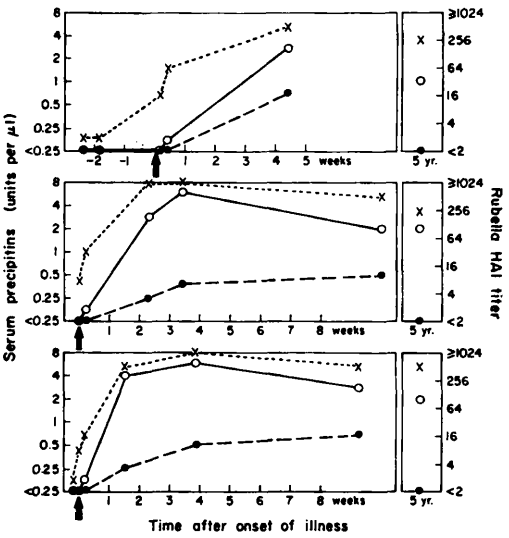

FIG. 2. Serum antibody responses over a 5-year period in 3 children with rubella. Hemagglutinin-inhibiting antibody, (×); anti-θ precipitin, (○); anti-ι precipitin, (●). Arrows indicate time of appearance of the rash. A point on the baseline (<0.25 units of precipitin/μl) means that no precipitate was visible. Where there was a trace of precipitin, but not amounting to 0.25 units/μl, the point is placed just above the baseline.

children with rubella. The presence of substantial amounts of anti-θ antibody, with little or no anti-ι, and with no change in either over the course of a few days, appears to signify previous infection with rubella virus and probable immunity.

Discussion. In cultures of cells from 3 different species, 2 precipitating antigens— θ and ι—appear following infection with rubella virus. In PS-RV cultures, both are found mainly in the liquid phase and only minimally, if at all, in adherent or floating cells, or in the characteristically abundant cellular debris. This suggests that they accumulate in the medium because of cell disruption, and/or continuing release from the surface of intact cells. There is as yet no indication whether θ or ι are structural components of the rubella virion (or their precursors); or nonvirion antigens whose synthesis is directly specified, or quite particularly derepressed, by the infecting virus. They do not seem to be related to "classical" Forssman antigen or to the sheep-cell antigen reacting typically with

the heterophile antibody of patients with infectious mononucleosis, for neither anti-ι nor anti-θ is absorbed by boiled guinea pig kidney or sheep red cells. The antigenic determinants are apparently associated with separate macromolecules, neither of which is sedimentable by forces that bring down rubella virus, or adsorbed on to chick red cells along with the hemagglutinin. How far θ, and in particular ι, can be equated with the small-particle complement-fixing antigen(s) described by several authors (15–22) has still to be determined.

Neither θ nor ι seems to be concerned, as such, with the HA function of rubella virus; but the HAI antibody response runs parallel with that of anti-θ precipitin, and a possible explanation would be that θ represents a precursor, or by-product, of the hemagglutinin—perhaps even an incomplete, "nonhemagglutinating HA." As for ι, its origin and function are as yet unknown.

Summary. Two specific precipitating antigens, *theta* and *iota*, have been detected by immunodiffusion of rubella virus reactants. They are subviral in size, and neither appears to be an active virus hemagglutinin. In patients with rubella, anti-θ precipitin rises promptly, along with antihemagglutinin, and can be detected for years, whereas anti-ι is made more slowly and declines sooner. The serum precipitins may reliably reflect the state of immunity.

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Note added in proof. After this paper had been submitted for publication, an article appeared, by N. J. Schmidt and B. Styk (J. Immunol., 1968, **101**, 210), reporting a positive immunodiffusion reaction with rubella "viral" and "soluble" antigen preparations; in some experiments a second precipitate was observed. The relationship of these antigens to θ and ι is at present under collaborative study.

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