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AUSTRALIA (HEPATITIS-ASSOCIATED) ANTIGEN: PHYSICO-CHEMICAL AND IMMUNOLOGICAL CHARACTERISTICS*

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I. INTRODUCTION

The search for etiological agents in hepatitis has had a long and checkered history. From clinical, epidemiological, and controlled hu-

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man transmission studies two types of transmissible, presumably viral, hepatitis have been defined. The first, infectious hepatitis (IH; Type A) has a relatively short incubation period (15–45 days, but usually about 30–35 days), and may be transmitted by either the fecal-oral or parenteral route. Numerous common source epidemics attributable to contaminated food, water, or milk have been described. Secondary infections among close contacts are common, in households and, particularly, in large institutions for mentally retarded children in which IH may become established as an endemic problem. The second type, serum hepatitis (SH; Type B) has a long and highly variable incubation period (45 to more than 100 days). Until a few years ago, SH was thought to be transmissible only by parenteral inoculation. Recent transmission studies and epidemiological observations, however, have provided good evidence that the infecting agent may also be acquired by the oral route, and, like IH, may become endemic in institutional settings (Krugman *et al.*, 1967).

This review deals with the discovery, characterization, immunology, and etiological significance of "Australia antigen," a new antigenic specificity appearing in the serum of patients with serum hepatitis, and carried on characteristic lipoprotein particles. In addition to its original name, given before its relationship to viral hepatitis became known, the antigen has also been given the following designations: Au(1), SH antigen, Au/SH antigen, hepatitis antigen, and hepatitis-associated antigen (HAA). At this time, there is no clear evidence that these terms refer to more than a single entity.

II. DISCOVERY AND EARLY STUDIES

A. Identification of Australia Antigen

Sera from persons receiving multiple transfusions have proved of value in the detection of serum isoantigens. Using this approach, and the Ouchterlony technique of two-dimensional double immunodiffusion, Allison and Blumberg (1961) and Blumberg *et al.* (1962) were able to define a new lipoprotein polymorphism, the Ag system. [Details of the original and later immunodiffusion methods will be discussed below (Section V, C)]. Continuing the search, Blumberg and his co-workers, in 1963, identified a new precipitating antigen with the help of serum from two multiple transfused hemophiliacs (Blumberg, 1964; Blumberg *et al.*, 1965). These sera contained an antibody which reacted with the serum of an Australian aborigine to give a single line of precipitation. The precipitating component, designated "Australia antigen," appeared to represent a single antigenic determinant, and was

clearly distinct from previously described human serum isoantigens. It was considered at first to be another example of a serum protein polymorphism. The possession of Australia antigen was held to distinguish the phenotype Au(1), reflecting the genotype Au^1/Au^1 . The allele Au^1 was postulated to be autosomal and recessive. Thus, individuals lacking Australia antigen [phenotype Au(0)] would be either homozygous Au/Au , or heterozygous Au^1/Au , the dominant Au allele acting to prevent synthesis or detection of the antigen (Blumberg *et al.*, 1966; Blumberg *et al.*, 1967a). This hypothesis of a simple genetic determination of Australia antigen was later modified, with the discovery of its peculiar association with leukemia, Down's syndrome, and hepatitis (Blumberg *et al.*, 1969). In the meantime, however, it had led to a notable series of studies by Blumberg and his associates (Blumberg *et al.*, 1965, 1966, 1967a,b), in which they looked for the antigen in various populations in different parts of the world.

B. Geographical Studies

Early serum surveys soon revealed an uneven distribution of Australia (Au) antigen in different human populations. The data shown in Table I are taken from Blumberg *et al.* (1968). Possession, or expression, of the antigen was relatively common among the peoples of South-eastern Asia and the Pacific Islands, as well as in Australian aborigines (2-13%). By contrast, its frequency in the general United States population was low ($\leq 0.1\%$).

C. Genetic Studies

The variation between different populations appeared, at first sight, to provide possible support for the hypothesis of the genetic control of the synthesis of the Au(1) macromolecule. Further studies of families were made to test the suggestion that the antigen might be specified by an autosomal recessive gene (Blumberg *et al.*, 1966). The data obtained were not sufficient to provide firm support for this thesis, although they did not rule it out.

Two later pieces of evidence combined to make the early simpler genetic interpretation less convincing: (1) it began to be apparent that environmental factors significantly affected the detectability of Au antigen in the serum, and (2) it was observed by Prince (1968a), and soon generally confirmed, that the duration of antigen in the serum was usually transient (a few days to several weeks), but occasionally it persisted indefinitely (Giles *et al.*, 1969; Zuckerman and Taylor, 1969). The simple presence or absence of a gene product was inadequate to explain these findings.

More recently, however, the hypothesis has been modified to state (1) that the persistent presence of Australia antigen in the serum, i.e., the Au(1) phenotype, reflects a peculiar individual susceptibility to the development of chronicity after infection with a variety of agents, including that of serum hepatitis with its specific association with Au

TABLE I
FREQUENCY OF AUSTRALIA ANTIGEN IN DIFFERENT POPULATIONS

Population	Location	Number tested	Number Au ⁺	Percent Au ⁺
Americas				
Eskimos	Alaska (U.S.A.)	394	1	0.3
Indians, Sioux	South Dakota (U.S.A.)	130	0	0
Afro-Americans	Georgia and Maryland (U.S.A.) ^a	607	0	0
Caucasians			0	0
Indians, Maya	Yucatan	1417	4	0.07
Indians, Cashinahua	Peru	89	18	20.2
Oceania				
Micronesians	Marshall Islands	474	34	7.2
Melanesians	New Guinea	166	6	3.6
Aborigines	Australia	1807	38	2.1
Asia				
Filipinos	Cebu, P. I.	764	37	4.8
Japanese	Japan	1034	5	0.5
Israelis	Israel	340	4	1.2
Africa				
Pare	Tanzania	120	1	0.8
Ghanaians	Ghana	95	9	9.5
Europe				
Finns	Finland	924	1	0.1
Greeks	Greece	857	15	1.8

^a In further surveys of hospital patients (serial admissions) and nonhospitalized "normal" controls in the U.S.A., 0.2% of the patients (2/1055), and 0.1% of the controls (2/2412) were positive for Australia antigen. Table modified from Blumberg *et al.* (1968).

antigen (*vide infra*); and (2) that it is this tendency toward development of a chronic carrier state which is affected by at least one autosomal recessive gene, still designated Au¹ (Blumberg *et al.*, 1969; Blumberg, 1970). The data in support of this expanded hypothesis were derived from studies of the island populations of Cebu (Philippines) and Bougainville (New Guinea) in which hygienic and sanitary conditions were such as to warrant the assumption that various orally transmitted infections, including both forms of viral hepatitis (Krugman *et al.*,

1967), would be endemic in the population so that there would be ample opportunity for exposure of all individuals, in time, to the infectious agents. The results, so far, do not appear to have disproved the hypothesis; but many more data from other presumably "hepatitis-saturated" populations demand analysis before this novel and provocative hypothesis can be accepted as nearing establishment.

D. Association of Australia Antigen with Different Diseases

In their studies of various groups within the United States, Blumberg and his colleagues (1965) observed early that Au antigen was unusually frequent in individuals with certain forms of leukemia. This association was later found to be true also for patients with Down's syndrome, lepromatous leprosy, and hepatitis (Blumberg *et al.*, 1967a,b). These workers further noted that among children with Down's syndrome, those in institutions showed a markedly higher frequency of Au antigen in their serum than those remaining at home (28 vs. 0%) (Sutnick *et al.*, 1968). Extending these observations, they and other investigators noted that the staff of mental hospitals and institutions likewise included more Au-positives than the general population. It became apparent that environmental conditions in such institutions were an important factor in determining the frequency of possession of Au antigen; and the suggested relationship with some acquired factor, or transmitted infectious agent, was strengthened by the finding of strikingly high proportions of antigen possessors among patients with diseases in which some depression or disturbance of immune function might be expected, e.g., leukemia and lepromatous leprosy (Blumberg *et al.*, 1968).

A more recent example of the tendency of nursing personnel, as well as patients, to acquire Au antigen is seen in the experience of chronic renal hemodialysis units (London *et al.*, 1969a; Turner and White, 1969).

III. ASSOCIATION OF AUSTRALIA ANTIGEN WITH HEPATITIS

The first indication of an unusually high prevalence of Au antigen in the serum of hepatitis patients was published by Blumberg *et al.* (1967a). Soon afterward, Prince (1968a), and Okochi and Murakami (1968) demonstrated the appearance of Au (or "SH") antigen in the serum of individuals, usually between 5 and 10 weeks after experimental inoculation or blood transfusion. In most cases, the antigen persisted for one to several weeks and then disappeared. But in 2 out of 7, and 1 out of 14 cases, respectively, it was still present in the latest serum tested (5-7 months after inoculation). Others have confirmed and amplified these findings, e.g., Hirschman *et al.* (1969), 4 of whose 46 Au-positive (Au+) cases of SH retained antigen for >10 months after onset; and

Giles *et al.* (1969), who reported persistence of Au for at least 3 years in 9 of 18 persons who received the MS-2 strain of SH agent. In these patients, the serum glutamic oxaloacetic or glutamic pyruvic transaminase rose to abnormally high levels, usually 5 to 15 days after the initial appearance of Au; but, thereafter, the correlation between elevated enzyme levels and serum antigen varied widely. In most of the cases with overt disease, symptoms began some 7–21 days after Au antigen first became detectable. These findings indicated a special temporal relationship of Au antigen with the SH form of viral hepatitis, as contrasted with its unchanging presence or absence in leukemia, Down's syndrome, and lepromatous leprosy.

The association of Au antigen with viral hepatitis has been confirmed in many laboratories. The principal point of disagreement has been the question of whether it is implicated in both forms of viral hepatitis, or specifically limited to the long-incubation SH form. Suffice it to say here that in those studies involving well-documented, experimentally produced infections, the antigen has been regularly found in all, or almost all, cases of SH, but has not been detectable in short-incubation disease of IH type (Prince, 1968a; Giles *et al.*, 1969; Le Bouvier *et al.*, 1969). Furthermore, in studies of several common-source outbreaks of IH type hepatitis, no Au antigen was demonstrable in any of the patients concerned (Prince *et al.*, 1970; Mosley *et al.*, 1970). It is noteworthy that none of a group of volunteers experimentally infected by mouth with the Akiba strain of IH (Neeffe and Stokes, 1945) developed detectable antigen. One individual, who already possessed Au antigen in his serum before inoculation, continued to do so throughout the course of his experimental infection. However, a possible result of infection with the IH-type agent, in this case, was a decline in the concentration of the already circulating Au antigen between the fourth and eighth weeks after inoculation. It remains to be seen whether this was a coincidental fluctuation, or causally related to the experimentally introduced agent, e.g., as the result of interference with Au antigen synthesis at the cellular level, or due to the blocking effect of a circulating inhibitor or cross-reacting antibody. Initial experiments to test the second possibility have so far yielded negative results (Le Bouvier *et al.*, 1969).

Thus, while Au antigen is consistently found in cases of long-incubation viral hepatitis, the significance of its reported presence in sporadic cases designated as IH has still to be determined. London *et al.* (1969b) noted that Au was rarely detected in acute phase sera obtained from children with hepatitis, a finding supported by subsequent studies reported by Prince *et al.* (1970).

Among other forms of acute and chronic liver disease, including drug hepatitis, biliary cirrhosis, alcoholic cirrhosis, postnecrotic cirrhosis,

and infectious mononucleosis, Au antigen is remarkably absent (Gocke and Kavey, 1969) with the exception of chronic active hepatitis in which Au antigen has been found, in the United States (Wright *et al.*, 1969; Gitnick *et al.*, 1969) and in Australia (Mathews and Mackay, 1970). However, it has so far not been found in such cases in England (Fox *et al.*, 1969), Denmark (Reinicke and Nordenfelt, 1970), or Chile (Velasco and Katz, 1970).

IV. PROPERTIES OF AUSTRALIA ANTIGEN

A. Introduction

The serological reactions of Australia (Au) antigen and antibody are dealt with separately in the following section (p. 374). This section considers other properties of the antigen, and of the particles which carry the Au specificity.

B. Chemical Properties

In early studies, Alter and Blumberg (1966) investigated the staining properties of Au antigen-antibody precipitates in agar gel: a strong reaction was obtained with the protein stain azocarmine; there was also a weak and variable affinity for the lipid stain, Sudan Black, but the intensity of the reaction was less than that given by the serum lipoproteins. The participation of protein in the reactivity of Au antigen has also been shown by its sensitivity to trypsin (2.5%, 37°C, 2 hours), and to treatment with sodium dodecyl sulfate and 2-mercaptoethanol (1% of each, 37°C, 1 hour) (Le Bouvier, 1969). Treatment with diethyl ether, or fluorocarbon, has effects on the morphology of Au-antigenic particles, which are discussed below (p. 369); but their antigenic specificity is not altered, and the level of activity remains unchanged, or may even be increased (Barker *et al.*, 1969).

There appears to be no report of the presence of carbohydrate or nucleic acid associated with the Australia particles and their antigenic specificity. If indeed there be any nucleic acid, it is evidently not present in a concentration comparable with that of the ribonucleic acid in "full," D-antigenic poliovirions (~25%); for when immunodiffusion bands of "full" (D) and "empty" (C) poliovirions, and Au antigen, were stained with acridine orange (cf. Cowan and Graves, 1968) the poliovirus D band stained and fluoresced brightly, whereas the C and Au bands remained completely unstained (Le Bouvier, 1969).

C. Physical Properties

Long before its particulate nature was known, Au antigen was suspected of having a high molecular weight (Alter and Blumberg, 1966),

since it was excluded from gels of Sephadex G-200. Moreover, the character of its precipitate band in agar indicated an antigenic particle, or macromolecule, distinctly larger than the presumably IgG antibody molecule with which it was reacting. Recent immunodiffusion studies have given a figure of $x_g \simeq 0.4$ for the equivalence position of Au antigen-antibody precipitates (i.e., the fractional distance between the antigen and antibody cups at which the precipitate forms when the reactants are meeting in optimal proportions). Here, the reacting antibody was known to be of IgG class. The diffusion coefficient, D_b , for the antibody was taken as 4.81×10^{-7} cm²/sec (cf., van Regenmortel, 1966). From the equation

$$x_b^2/x_g^2 = D_b/D_g$$

where $x_g = 0.4$, and $x_b = 1 - x_g$, the diffusion coefficient for Au antigen, D_g , was calculated to be $\sim 2 \times 10^{-7}$ cm²/sec (Le Bouvier, 1969).

In early studies of adsorption chromatography on DEAE-cellulose, Au antigen was eluted by 0.1–0.2 M phosphate, pH 7.0, after IgG, and together with, or slightly ahead of, β -lipoprotein, IgA, IgM, and albumin (Alter and Blumberg, 1966). When subjected to immunoelectrophoresis, Au antigen somewhat resembled in mobility an α_1 -globulin (Alter and Blumberg, 1966).

The buoyant density of the antigen-bearing particles is intermediate between that of serum lipoproteins and most other proteins. In the first studies, done by flotation in KBr, the antigenic activity was associated with fractions of density >1.063 and <1.30 gm/ml (Alter and Blumberg, 1966). In recent studies, there has been general agreement about the intermediate density, but some discrepancy in the precise figures: in CsCl, the maxima were 1.20 (Gerin *et al.*, 1969), 1.21 (Le Bouvier and Hierholzer, 1969; Millman *et al.*, 1970a), and 1.24 gm/ml (Barker *et al.*, 1969); in sucrose, 1.16 (Gerin *et al.*, 1969) to 1.18 (Le Bouvier and Hierholzer, 1969); and in potassium tartrate, 1.15 (Gerin *et al.*, 1969). After treatment with ether, fluorocarbon, or sodium deoxycholate, the density increases, e.g., in CsCl, from 1.20 to 1.22 or 1.23 (Gerin *et al.*, 1969), or from 1.24 to 1.27 or 1.28 (Barker *et al.*, 1969); or from 1.18 to 1.20–1.21 in sucrose (Le Bouvier and Hierholzer, 1969). In one study (Gerin *et al.*, 1969), freezing and thawing, or treatment with Tween 80 of an Au antigen fraction, which had been twice banded in CsCl and then separated by rate sedimentation in sucrose, caused a fall in buoyant density from 1.20 to 1.18 in CsCl, and also resulted in the emergence of a "significant amount" of Au+ material with a density of 1.39 gm/ml. It is not yet known what this represents; but its density would be compatible with that of a nucleoprotein.

The sedimentation coefficient of Au-antigenic particles has been reported as 110 S in one study, in which adeno-associated virus (AAV-1), with $S_{20,w} = 104$, was used as a marker (Gerin *et al.*, 1969). The determination was made in sucrose (5–20% w/w, 39,000 rpm, 3 hours), using as test material Au antigen that had been purified by two sequential equilibrium centrifugations in CsCl density gradients. An identical sedimentation coefficient of 110 S was found by Prince (1970). In another study done under similar conditions, but using serum as the material under test (Le Bouvier, 1969), the S value of the Au antigenic activity was found to be intermediate between those of IgM (19 S) and "empty" poliovirus capsids (73 S). The reasons for these apparent discrepancies have still to be discovered.

Where the material under examination consists of a preselected segment of the population of Au-antigenic particles, e.g. the peak fraction from a CsCl isopycnic centrifugation, the values obtained for buoyant density and sedimentation coefficient may fall within quite a narrow range. But when the test material is untreated, unheated serum, these values show a greater spread, and sometimes more than one peak, suggesting considerable particle heterogeneity (Figs. 1 and 2).

It is clear that the degree of aggregation of Au-antigenic particles will affect the determination of their size, sedimentability, and diffusion characteristics, and likewise their capacity to react in serological tests (*vide infra*, p. 374). Purcell *et al.* (1969) have reported decreases of Au antigenic titer of up to 50%, as detected by immunodiffusion, after heating at 56°C for 30 minutes; this could be caused by aggregation without degradation, since the complement-fixing antigen was not destroyed by overnight incubation at this temperature (Gerin *et al.*, 1969). Other results have indicated no appreciable loss of antigenic activity, as tested by immunodiffusion, after heating at 56°C for 1 hour (Millman *et al.*, 1970a), or after 20 minutes at 59°C, or more than 1 year at room temperature (Hierholzer *et al.*, 1970). Gerin *et al.* (1969) reported a marked drop in Au antigen titer in some experiments, when Au antigen which had been fractionated by density in CsCl was subjected to velocity sedimentation in sucrose; and they pointed to particle aggregation as the probable reason for this loss. The complement-fixing activity of Au antigen was not affected, however, by incubation at pH 2.7 for 3 hours at room temperature, or by exposure to ether (20% v/v), either overnight at 4°C, or for 4 hours at room temperature.

D. Morphology

As the peculiar geographical distribution and disease associations of Au antigen began to be appreciated, the question arose whether the

antigen might be related to, or might itself be, a transmissible agent and, in particular, a virus. Bayer *et al.* (1968) therefore examined in the electron microscope, by the technique of negative staining with sodium silicotungstate, Au+ fractions derived from a sample of serum which had been separated in a sucrose density gradient and then electrophoresed on cellulose acetate. They made the astonishing dis-

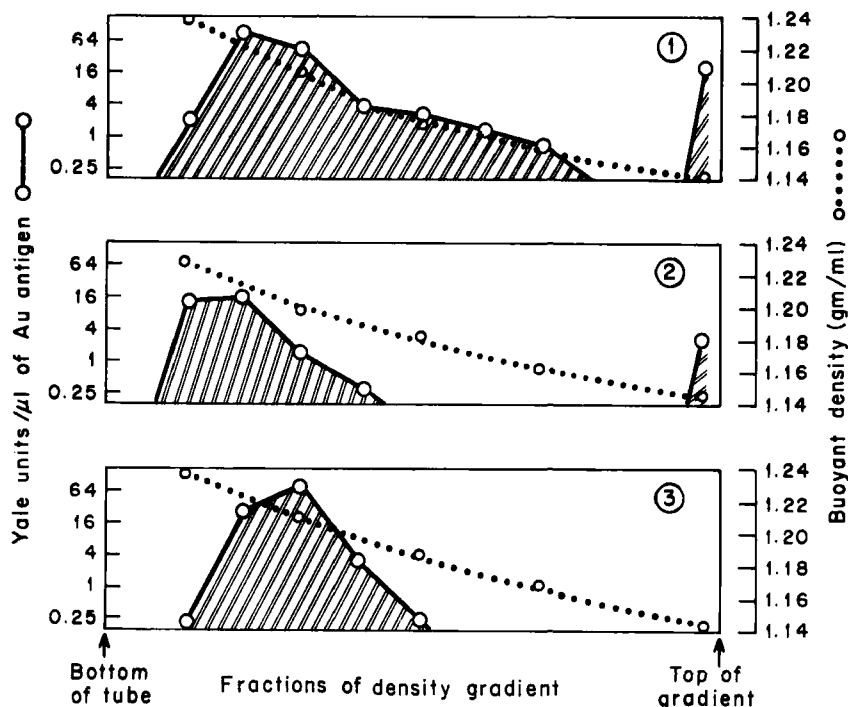


FIG. 1. Isopycnic centrifugation in cesium chloride of Au antigen in 3 different sera. Serum, 1 ml, mixed with 3.8 ml of 26% (w/w) CsCl ($\rho \approx 1.24$); spun at 37,000 rpm for 21 hours at 5°C in Spinco SW 50 rotor; fractions of ~ 0.5 ml collected, and buoyant densities determined by weighing 100- μ l aliquots.

covery of round and ovoid particles, 20 nm in diameter, possessing suggestive knoblike surface projections 3 nm in diameter. Some particles seemed to have a central 7-nm diameter core, while a few appeared to be "empty" shells filled with the stain. The particles were not seen in a heavier fraction lacking Au antigen, or in fractions of corresponding density derived from Au-negative sera. Furthermore, aggregates of these particles could be seen electron microscopically in mixtures of the Au+ fractions with rabbit antisera containing anti-Au precipitin.

These findings have been repeatedly confirmed by other workers (Figs. 3 and 4). In addition to the spherical and ovoid particles, tubular forms have been observed, their frequency varying from one serum

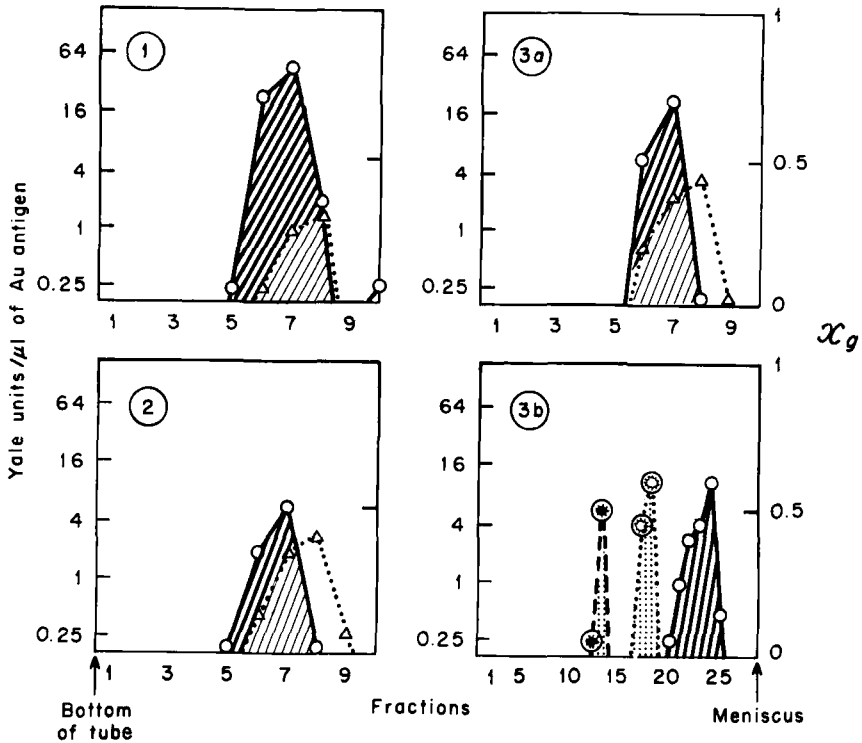


FIG. 2. Velocity sedimentation in sucrose of Au antigen and IgM in 3 different sera (parts 1, 2, and 3a). Serum, 0.25 ml, layered on 4.5 ml of a 10–30% (w/w) sucrose gradient; spun at 35,000 rpm for 2.5 hours at 5°C in Spinco SW 50 rotor; 0.5-ml fractions collected. Serum No. 3 was also rerun in parallel with poliovirus “full” (D) and “empty” (C) capsids (3b). Serum, 0.2 ml, or poliovirus concentrate (D + C), layered on 4.5 ml of 9–45% (w/w) sucrose gradient; spun at 30,000 rpm for 2.5 hours at 5°C in Spinco SW 50 rotor; 3-drop fractions collected. O—O = Au antigen; Δ·····Δ = IgM; ●---● = poliovirus D antigen; ⊙····⊙ = poliovirus C antigen. Concentrations of the poliovirus antigens, and of IgM, have not been measured in units or milligrams, but are given as a function of x_g , the fractional distance between antigen and antibody cups at which precipitation is taking place (cf. p. 377).

to another (Almeida and Waterson, 1969). In these forms, an appearance of regular transverse striation can often be made out, with a periodicity of ~3 nm (Almeida and Waterson, 1969; Almeida *et al.*, 1969). These tubules may attain lengths of <50–200 nm or more, often having

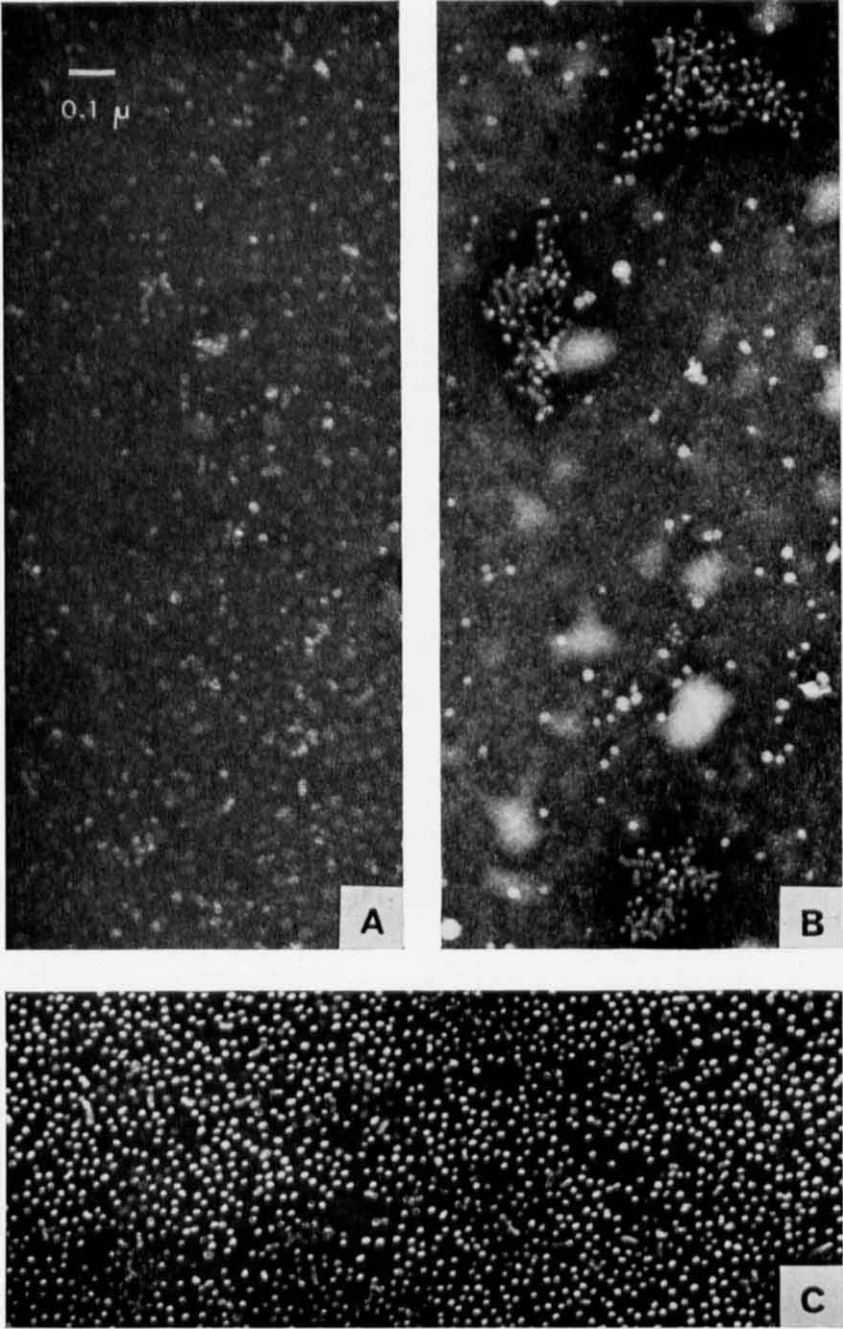


FIG. 3

variably wider rounded swellings, or bulbous formations, at one or both ends, and may exhibit sharply angled and even right-angled bends, as well as a lesser degree of smoother curvature (Bayer *et al.*, 1968; Almeida and Waterson, 1969; Almeida *et al.*, 1969; Zuckerman, 1969).

Barker *et al.* (1969) have studied the size distribution of the particles present in Au+ fractions from CsCl density gradients, both before and after treatment with ether or fluorocarbon (Genetron). The figure from their paper, with modifications, forms the basis of Fig. 5. It shows the larger modal particle diameter (20 nm) and broader spread of particle size in the untreated preparation. After treatment, the particles appeared smaller (modal diameter 16 nm), more sharply outlined and more evenly dispersed; there was a moderate increase in their buoyant density (*vide supra*); and virtually all the tubular forms had disappeared. The Au antigen titer, as measured by complement fixation, was, if anything, somewhat higher after the extraction. Barker *et al.* interpreted their findings to mean that an outer lipid coat ~ 2 nm thick, and in some cases probably also attached anti-Au antibody (Shulman and Barker, 1969), had been removed by the extraction.

Since treatment with lipid solvents did not appear to modify the antigenic specificity, it would seem that the putative lipid coat does not completely cover the particle surface. A further possibility might be that the bulk of the lipid is present as an interparticulate "cement," and that the larger rounded and ovoid particles are in fact made up of two or more "unit particles" (monads); some, at least, of the elongated forms

FIG. 3. Electron micrograph of Au particles from serum of a patient with post-transfusion hepatitis. Magnification: $\times 62,000$. (We are indebted to Dr. Virginia Killby for the electron microscopic studies.) A. Au particles from untreated serum, negatively stained with 2% potassium phosphotungstate, pH 5.9. The serum was centrifuged at 35,000 rpm for 5 hours at 5°C in a Spinco SW 50 rotor, and the pellet resuspended at $\sim 100\times$ concentration in 0.15 M NaCl, 0.05 M Tris, pH 7.6. Note spherical and ovoid particles of diameter ~ 20 nm, with a few short tubular forms. The length of the bar is equivalent to 100 nm. B. Au particles agglutinated by anti-Au antibody. The same suspension as that seen in Fig. 3A was mixed with antibody-containing serum from a multitransfused patient, held at 4°C overnight, and examined by negative staining, as described above. Note aggregates of particles, which also include an occasional tubule. C. Au particles, concentrated and partially purified. The serum was fractionated by two sequential equilibrium centrifugations in CsCl density gradients (*cf.* Gerin *et al.*, 1969). The final Au antigen-containing fractions were diluted with 15 volumes of Tris-NaCl buffer, and the particles sedimented at 39,000 rpm for 4 hours at 5°C in a Spinco SW 50 rotor. The pellet was resuspended in buffer to give a concentration of ~ 100 -fold with respect to the original volume of serum. A droplet of the particle suspension was examined by negative staining. In addition to the characteristic rounded particles, occasional short tubules are seen, as well as "empty" particles into which the stain has penetrated. (We are indebted to Dr. Walter Hierholzer for this preparation.)

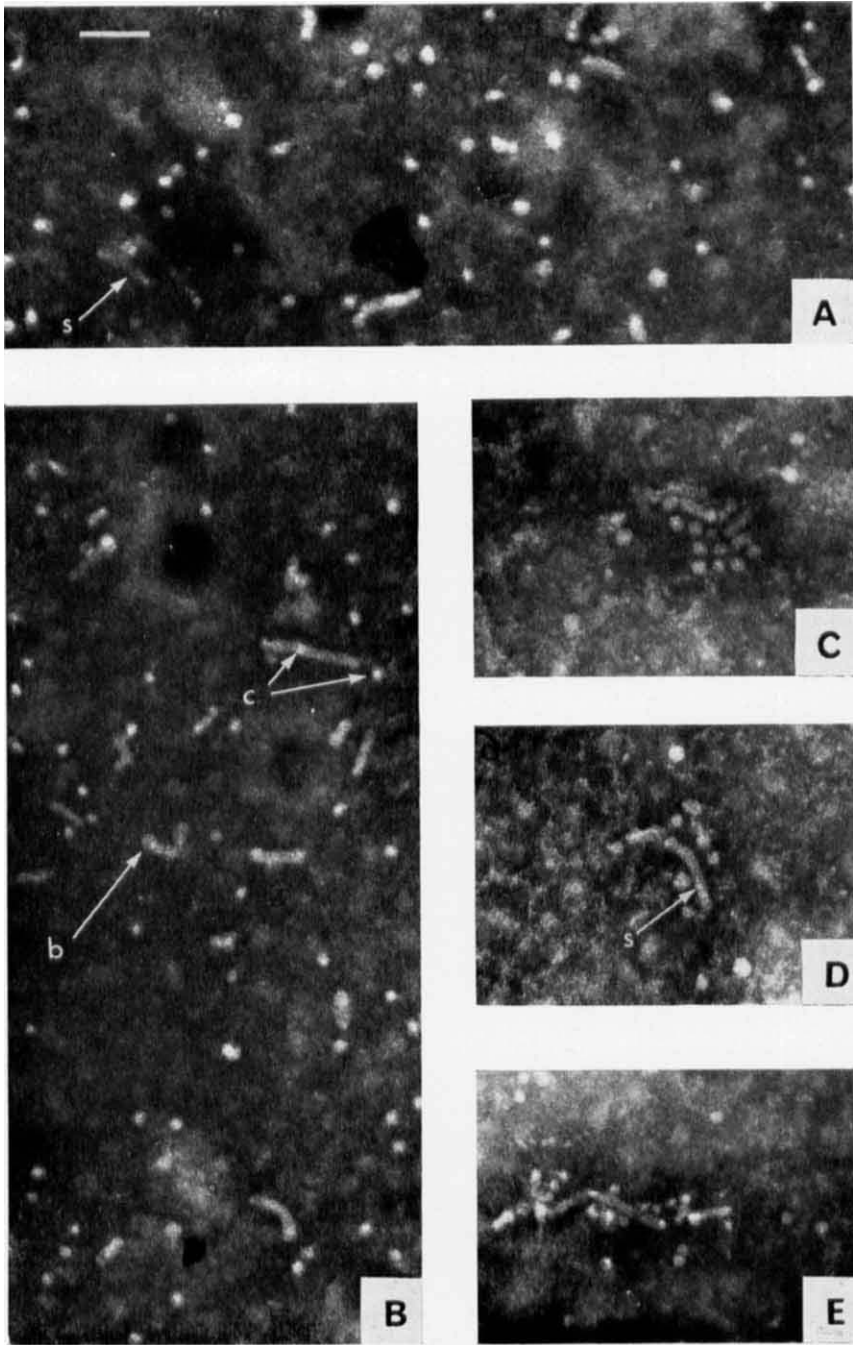


FIG. 4

might consist of strings of such monads, while others would be relatively "empty" lipoprotein sacs, possibly containing terminal and occasional intercalary monads. Based on this hypothesis, the lipid would be dissolved upon exposure to ether or fluorocarbon, and these larger structures—dyads, triads, etc., as well as the tubular pleiads—would be dismembered into their component monads.

A second class of particles has been observed in the serum of Au+ hepatitis patients by Dane *et al.* (1970). Of 16 patients whose serum contained 22-nm particles, 3 also showed lesser numbers of more complex, virus-like particles, ~42 nm in diameter, consisting of an inner body of 28-nm diameter, with a 2-nm shell, and an outer coat ~7 nm thick. The large and small particles formed mixed aggregates when treated with anti-Au sera, suggesting the sharing of a surface antigen, and the possibility that the larger form may be the SH virus, while the smaller particles and tubules may represent surplus virus-coat material. The finding of larger particles in certain Au+ sera has been confirmed by Cossart and Field (1970) and by Gust *et al.* (1970).

E. Biological Properties

The nature and significance of the Australia particles pose the biggest single question, at present, in the microbiology of hepatitis. Their surprisingly "viruslike" appearance has frequently led to the tacit assumption that these are indeed the virions responsible for the infection. The extreme opposite view, suggesting that they solely represent specifically modified, neoantigenic cell fragments produced in response to infection with an as yet undiscerned virus, will be considered later (p. 390). Also to be considered will be the view that the particles, while related to the causal virus, are not for the most part whole virions, but analogous, rather, to the envelopes of the myxoviruses, and composed of unaltered host-cell material as well as of virus-specified or virus-modified molecules, the great majority of these envelopes being

FIG. 4. Electron micrographs of Au particles from serum of a patient with chronic anicteric hepatitis. Magnification: $\times 92,000$. (By the courtesy of Dr. Virginia Killby.)

A and B. Au particles from fraction No. 7 of the rate zonal sedimentation experiment illustrated in Fig. 2, part 1, examined by negative staining as indicated for Fig. 3. Note rounded particles ~20 nm in diameter, and tubules which are both more numerous and longer than those seen in Fig. 3, some showing sharp angulations. Note also the appearance of "cores" (c) in some spherical particles, and of cores and "bulbs" (b) in the elongated forms. An occasional tubule shows ill-defined, but apparently regular, transverse "striation" (s). The length of the bar in Fig. 4A is equivalent to 100 nm.

C, D, and E. The same fraction No. 7 mixed with serum containing anti-Au antibody. Note aggregates made up of both rounded and tubular Au particles. The tubule in Fig. 4D shows cross-striation (s).

“incomplete” or “empty,” and different in structure, though presumably not markedly in size, from the infective particles (cf. McCollum, 1952; Barker *et al.*, 1970).

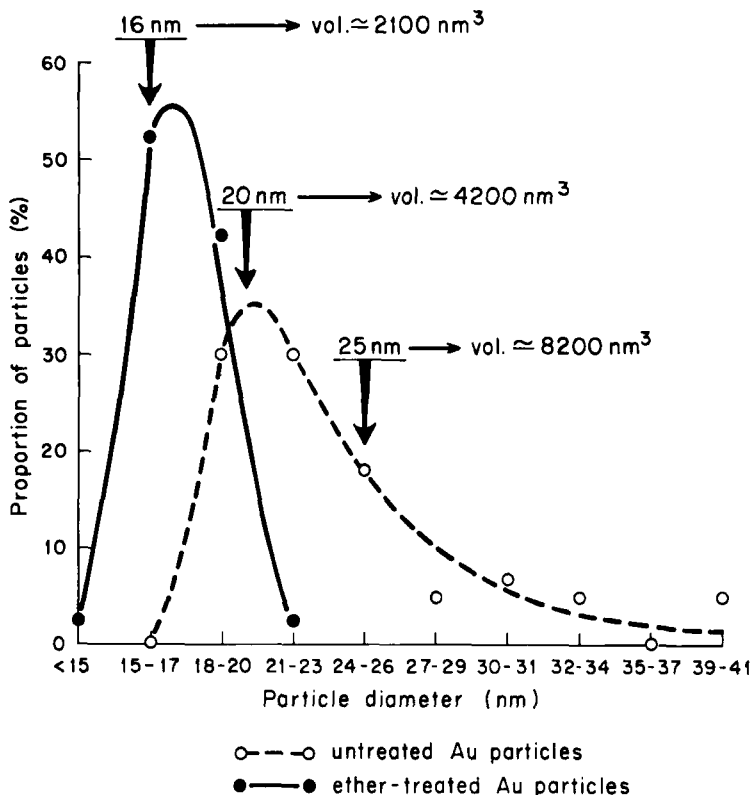


Fig. 5. Effect of ether treatment on the size of partially purified Au-antigenic particles (Barker *et al.*, 1969). Au-positive serum was passed through a Celite filter and fractionated by centrifugation in a CsCl gradient. Au⁺ fractions were examined electron microscopically, by negative staining with phosphotungstic acid, before and after exposure to ether (equal parts, 1 hour, 0°C). Volumes are those calculated for spherical particles of the given diameters. On this basis, close to 40% of untreated Au particles, with diameters of 25-40 nm, have volumes ranging from 8000 to 34,000 nm³, *i.e.*, 4 to 16 times the volume of the 16 nm-diameter “monadic” particle. (Figure modified from Barker *et al.*, 1969.)

The Au-antigenic particles must evidently attain impressive concentrations in the serum to be detectable so readily, whether as particles by electron microscopy, or as antigen reactants by immunodiffusion. By comparison with the corresponding concentrations required for poliovirus, one may calculate that individual sera must often contain up to

10^{13} particles—or ~ 40 μg —per milliliter of serum. There appear to be no published reports, as yet, of particle counts; but one serum examined was found to have $\sim 10^{12}$ particles/ml (Melnick, 1969).

The tubular forms offer an additional ground for regarding Au-antigenic particles as conceivably related to some sort of “micromyxovirus.” The proportion of such forms appears to vary considerably from one individual to another, and likewise within a given individual at different times. Almeida and Waterson (1969) have put forward the suggestion that the tubules represent a more mature form of the infective agent, elaborated particularly in patients with chronic hepatitis, or in asymptomatic persistent carriers; while the spherical, ovoid, and small pleomorphic particles would represent a less well organized form of the agent. The question of the significance of these different structures in different types of patient will be taken up again (p. 390).

The infectivity of particle concentrates *per se* has not been tested directly in human subjects; but previously identified icterogenic sera have been found to contain Au antigen. Inoculation of various primates, and attempts to produce detectable infection of a wide variety of cell and organ cultures, are currently being undertaken in various laboratories, but no findings have yet been published. Both Au antigen and morphologically characteristic particles have been found in occasional individuals of several primate species, including the chimpanzee, the orangutan, and the gibbon (Hirschman *et al.*, 1969). Serial serum specimens obtained, during earlier transmission attempts, from chimpanzees inoculated with materials known to contain Au antigen, have been tested by immunodiffusion. No evidence of circulating Au was detected (McCollum, 1970).

Au antigen has been reported in the liver cells of patients with hepatitis by means of immunofluorescence tests with a fluorescein-labeled IgG fraction from an anti-Au rabbit antiserum (Millman *et al.*, 1969). Liver biopsies from 9 patients were examined: 4 had some form of viral hepatitis, including one chronic anicteric, and all had Au antigen in their serum; the other 5 were controls without liver disease, except for one with hepatoma. The biopsy tissue was minced in culture medium, and drops of the resulting cell suspension were spread on slides, air-dried, and fixed with acetone. Discrete fluorescent granules were seen within or on the nuclei (1–30 per nucleus) of almost every cell in the biopsies from the 4 hepatitis patients; they were absent in the controls. The specificity of the reaction was shown by a blocking test with the unconjugated anti-Au antiserum: subsequent addition of labeled antibody produced no fluorescence. (See also Section V, F.)

Other tissues examined included sternal bone marrow from 14 Au+ Down's syndrome patients, and kidney, jejunum, and mesenteric lymph

nodes from the chronic anicteric hepatitis patient referred to above. In none were the fluorescent granules detected.

Recently, Nowosławski *et al.* (1970) reported studies of liver cells obtained at autopsy from 12 patients with lymphoproliferative disorders. Six, whose serum contained Au antigen, all showed specific nuclear and/or cytoplasmic immunofluorescence, and also 20-nm diameter particles, sometimes in chains, within the nuclei. The other six, with Au-serum, showed neither specific immunofluorescence nor intranuclear particles.

V. SEROLOGY OF AUSTRALIA ANTIGEN

A. Introduction

The method used for the original detection of Au antigen and antibody in serum, and still perhaps most widely used, is that of two-dimensional double immunodiffusion (ID). Recently, other serological techniques, with different attributes of sensitivity and specificity, have been turned to advantage in this connection, viz., complement fixation (CF), immunofluorescence (IF), reversed passive hemagglutination (RPHA) of antibody-coated red cells, immune electron microscopy (IEM), immunoelectroosmophoresis (IEOP), and radioimmunoassay (RIA).

By virtue of their first identification and characterization, Au antigen and antibody are defined in terms of the precipitating reactants used by Blumberg and his colleagues, to which all subsequent reactants have been referred. In like manner, when doubt or discrepancies arise during the use of other serological procedures, these demand resolution by comparison with the results obtained by ID.

B. Reactants

Antigen reactants have consisted principally of sera, or fractions derived therefrom; but also of cells, either naturally infected or experimentally exposed. Antibody reactants have comprised (1) human sera; and (2) sera from animals hyperimmunized with whole serum, or fractions, containing Au antigen. Human sera with anti-Au precipitins have come from multiple transfused patients (e.g., with hemophilia or various kinds of anemia), and from patients on hemodialysis because of chronic renal disease. In the course of their periodic transfusions, these patients presumably received repeated, and perhaps rather small, stimuli of Au antigen, with the result that some reacted to it as an immunizing protein rather than as an infecting agent, and responded with antibody instead of antigen formation. Animals used have included rabbits, guinea pigs, and mice.

Rabbits (Melartin and Blumberg, 1966) were given an intramuscular injection of alum-precipitated Au+ serum, followed by a similar injection 2 weeks later, and an intraperitoneal injection after a further 10 days. Serum was collected 7–10 days after the third injection. After a two week rest, the immunization schedule was repeated. The resulting antisera required absorption with Au– human serum to remove antibodies against normal components.

Guinea pigs and rabbits (Purcell *et al.*, 1970) were immunized with Au+ serum fractions separated by two CsCl density gradient centrifugations followed by a rate sedimentation in sucrose. The antigen preparation was emulsified with Freund's complete adjuvant (1:1) and injected into the footpads. Boosting injections of aqueous immunogens were given subcutaneously at 1- to 2-weekly intervals, starting 4–6 weeks after the initial injection. Of the animals that received purified Au fractions, rabbits responded with poor anti-Au antibody formation, and also developed low levels of antibodies against normal serum components; guinea pigs, however, produced good levels of seemingly unispecific anti-Au antibody, with no response to other serum components.

Ascitic fluids have been obtained from immunized mice with or without the aid of ascites tumor cells. Hierholzer (1969), in this laboratory, has injected mice intraperitoneally with Au+ fractions prepared as described for guinea pigs, mixed 1:1 with Freund's complete adjuvant, followed by similar booster injections into the footpads 2, 4, and 6 weeks later. At 6–8 weeks, the mice were given $\sim 10^6$ ascites tumor cells (Sarcoma 180/TG). The ascitic fluid was tapped 1 week after the injection of tumor cells, and thereafter every 2–3 days for ~ 2 weeks, at which time the mice died of the tumor. Moderate levels of anti-Au antibody were detected by CF, and by ID, after the second booster injection. The fluids so far obtained have usually contained low levels of non-Au antibodies and have required absorption with normal human serum.

Millman *et al.* (1970b) produced immune ascitic fluids by the injection of Au+ serum and Freund's complete adjuvant, without tumor cells. The proportions were 3 parts of adjuvant to 1 of serum (later reduced 2:1) in the first intraperitoneal injection, and 1:1 in the second given 6 weeks later. Over half the mice developed ascites 2–4 weeks after the second injection, and the rate of fluid production could be increased by periodic intraperitoneal injection of a saline-adjuvant mixture. To absorb nonspecific antibodies, fluids were mixed with normal human serum, or else were tested in wells from which normal serum had previously been allowed to diffuse into the agar ("in-well absorption"). Of 111 sera judged Au+ with a human antiserum, 84 also reacted with ascitic fluid; besides the 27 sera that were Au+ only

with human antiserum, there were 6 others which reacted only with ascitic fluid. Some Au+ sera gave two precipitate bands with several of the mouse fluids, yet only one with human or rabbit antisera. Treatment of the ascitic fluid with 2-mercaptoethanol, as well as exclusion chromatography on Sephadex G-200, indicated that an appreciable part of the mouse antibody was apparently of IgM class. When fluids giving two bands were treated with mercaptoethanol, one of the bands was no longer seen. The suggestion of serological nonidentity between the human and certain of the mouse antibodies is discussed below (p. 385).

C. Immunodiffusion

The two-dimensional double diffusion procedures are based on the method of Ouchterlony (1958). Blumberg and his co-workers have used Oxoid Ionagar, 0.9% (w/v) in 0.07 *M* sodium phosphate, pH 7.4, with incubation of the test at 20°C for up to 1 week. Prince (1968b), to increase the sensitivity of antigen detection, modified the technique by using 1% agarose, made up in 0.1 *M* NaCl buffered with 0.01 *M* tris (pH 7.6 at 25°C), and adding 0.1% protamine sulfate and 0.001 *M* ethylenediaminetetraacetic acid to the gel. Protamine sulfate can cause clouding of the gel and occasional formation of obscuring haloes and opacities, and has accordingly been omitted in some laboratories. In our experience, the single most important factor in improving the detectability of tenuous precipitate bands was the use of a clear agarose in concentrations not exceeding 0.6% (w/v). The exact buffer used appeared to be less critical, except that it was preferable to exclude phosphate: we have used 0.1 *M* NaCl, buffered at pH 7.6 (25°C) with 0.1 *M* tris, and containing 0.1% (w/v) sodium azide as a preservative.

The questions of standardization and measurement, as is not unusual in the virological applications of ID, have scarcely been broached, let alone settled. Blumberg and his colleagues have employed the tube method of Preer and Preer (1959) to quantitate the Au antigen concentrations in their test sera. Unknown sera are tested in parallel against undiluted standard antiserum, and their content of Au antigen is expressed in Preer units, depending on the distance of the precipitate from the antigen-agar interface at a fixed time. For this purpose, the column of agar in the reaction tube is divided into 100 units.

In our laboratory, as a provisional method of assay, the roughly quantitative approach used for the measurement of poliovirus and rubella virus antigens (Le Bouvier, 1959) has been applied to the estimation of Au antigen and antibody. One serum pool containing Au antigen, and one containing anti-Au antibody, were selected as the reference standards. The position of equivalence for the Au system

was found to be ~ 0.4 of the distance from the Au antigen cup to the homologous antibody cup. The positions of the leading edges of precipitates formed by various interacting pairs of antigen and antibody dilutions were measured from photographs, and standard curves were drawn. The highest dilution of the standard antibody reactant still giving a distinct band of precipitate in the equivalence zone, with any dilution of the standard (or any other Au-specific) antigen reactant, was defined as containing 1 "Yale unit" of anti-Au precipitin per μl . The particular dilution of the antigen preparation with which it gave this precipitate was taken to contain 1 "Yale unit" of Au precipitating antigen per μl . Unknown reactants are compared with the standards, and their antigen or antibody content is similarly expressed in Yale units per μl .

The strength of Au precipitating antigen has also been measured by making serial dilutions of the sera under test, and determining, as the titer, the highest dilution which still gives detectable precipitation with an undefined, but relatively high, concentration of the antibody reactant.

Factors which may help to enhance the sensitivity of ID tests include: the utmost possible clarity of the gel; a reasonably close spacing of the cups; the use of larger cups for the sera under test which are suspected of having minimal levels of antigen or antibody; prefilling of cups with the unknown reactants 1-4 hours before disposition of the standard reactants, at which time the test cups are refilled; the use of standard antigen and antibody reactants in approximately equivalent proportions, and at concentrations which are (1) adequate to give a clear arc of precipitate whose ends approach the cups containing the unknown reactants, but (2) not so strong that weak reactions given by the unknown sera are "forced back" into their cups.

The pattern we have adopted has been the familiar hexagonal arrangement, or a series of "fused hexagons" which becomes, in effect, a middle row of alternating cups of antigen and antibody standards, flanked by two rows of cups containing the unknown reactants.

D. Complement Fixation

The greater sensitivity of CF, as compared with ID, led to its use in the study of the Au system. In most kinds of CF tests, the unknown material is tested with both a specific positive reactant and an appropriate negative control—antigen or antibody, as the case may be. In the Au system, where the antigen being sought is present among a rich assortment of normal serum components, it is necessary to know that the standard antibody reactant will not fix complement with any of

these other antigens. Human sera containing anti-Au antibody may not be the safest of reactants for this purpose; for it is clear that many, if not all, such sera from repeatedly stimulated donors may contain a variety of antibodies against different serum components. Animal hyper-immune antisera would seem to be preferable; and ideally, control anti-serum should also be prepared against an early Au- serum specimen taken from the same individual who provided the Au+ serum which served, either as such or after fractionation, as the source of immunizing antigen.

The types of CF tests which can be used for the detection and measurement of Au antigen, or antibody, include: (1) relatively simple "linear" tests in which dilutions of the unknown serum are tested with an optimal concentration of known anti-Au antibody and a selected dose of complement (cf. Purcell *et al.*, 1969); (2) two-dimensional tests of the standardizing "chessboard" type, in which serial dilutions of both antigen and antibody reactant are tested against each other, using a single dose of complement; (3) tests of "quantitative CF," in which the unknown serum is tested in dilutions against a single optimal concentration of known serum—either antigen or antibody source, as the case may be—and in which the amounts of complement fixed are determined (cf. Shulman and Barker, 1969); or (4) three-dimensional tests in which the concentrations of antigen, antibody, and complement are all varied. From (3) or (4), curves can be drawn of the total amount of complement fixed by varying combinations of the standard and unknown reactants. For convenience, the strength of an unknown serum so tested may also be given as the highest dilution which fixes a chosen dose of complement, e.g., two 100% hemolytic units, in the presence of optimal standard antigen, or antibody, as the case may be.

Whereas an ID test may be set up so as to detect both antigen and antibody reactivity in each unknown sample, a CF test is of course usually designed to look only for one activity or the other.

While ID tests have the advantage over CF tests of confirming or refuting *ab initio* the Au identity of any antigen or antibody detected in an unknown serum, they are inherently less sensitive, the disparity in sensitivity increasing with the size of the reacting particle which must diffuse into the gel in the ID test. In the comparative study of Purcell *et al.* (1969), the linear CF test employed was about 24 times more sensitive than ID in detecting Au antigen, but no more than 4 times more sensitive in detecting antibody. They used a modified Prince (1968a) method of ID, in which protamine sulfate was omitted from the gel. In Shulman and Barker's (1969) comparison, their complement consumption test was 200–300 times better than their ID procedure for the de-

tection of Au antigen, a quite remarkable degree of superiority. In our own series (Hierholzer and Le Bouvier, 1969), which employed a linear CF test with two 100% units of complement, the difference was ~16 times in favor of CF for detecting Au antigen; but there was at most only a 2 times difference in the ability to detect anti-Au antibody, and sometimes the ID method appeared to be slightly (~2 times) more sensitive than CF for this purpose.

Perhaps the greatest success of CF in the serological study of SH infection has been the identification, by Shulman and Barker (1969), of a transient phase in the later part of the incubation period, in most cases, during which the patient's serum becomes anticomplementary (AC). This AC activity rises to a peak from 4 to 6 weeks after infection, and then declines, at the same time as free Au antigen is first becoming detectable in the serum. In some cases, a second AC phase is seen after free Au antigen ceases to be detectable in the serum; and sometimes anticomplementariness is the only serological anomaly observed. It was suspected that this apparent AC effect might in fact be due to actual fixation of complement resulting from the simultaneous presence of circulating antigen and antibody, and the formation of immune complexes. This was confirmed by the finding that addition of further known Au antigen, or antibody, to the serum in question produced a situation of antigen or antibody excess, with a consequent prozone of diminished CF. The Au-specific character of the AC activity was further shown by fractionating such sera on CsCl gradients and finding Au particles apparently coated with antibody in the AC fractions. Treatment of these fractions with fluorocarbon, which dissociates virus-antibody complexes, reduced or eliminated their AC activity, and often doubled their Au-antigenic activity detectable by the addition of known antibody.

It is evident that CF is a good and sensitive means of looking for Au antigen, especially in experimental systems in which known antigen and antibody reactants are being manipulated, and the consequences assessed. For the detection of weakly antigen-positive sera it also comes into its own; but here there may be occasional situations in which the unispecificity of the reaction may need to be corroborated by ID, and perhaps by further CF cross-testing after absorption of the standard antiserum with the standard and the unknown antigen reactants.

E. Immune Electron Microscopy

The presence of Au antigen-antibody complexes in the serum of patients with hepatitis has also been demonstrated electron microscopically by Almeida and Waterson (1969), using the technique of negative staining with phosphotungstic acid (*vide supra*, p. 369). They described

their findings in 3 different types of individual: (1) a chronic, asymptomatic carrier of Au antigen, in whose serum randomly distributed spherical and tubular particles were seen, with no sign of aggregation and no evidence of antibody molecules attached to the particles; (2) a patient with "chronic active hepatitis," who possessed many pleomorphic and tubular, as well as spherical, forms, and in whom both single particles and aggregates were present, some of them apparently comprising several hundreds of particles; (3) a fatal case of acute hepatitis, whose serum contained very few elongated forms, but many characteristic spherical particles, all coated with large amounts of antibody and forming numerous aggregates, some of quite small size.

The symptomless carrier exhibits the condition of persistent, apparently tolerant, carriage of Au particles, without any production of anti-Au antibody. The chronic active hepatitis case evidently possesses Au antigen in excess, but has succeeded in elaborating a minute amount of antibody. In the fulminating case, it is apparently the anti-Au antibody that is present in excess.

The authors discuss the possible significance of these diverse relative proportions of Au antigen and antibody in these dissimilar types of case. They suggest an analogy between acute SH and serum sickness, which has been shown to be caused by immune complexes present in the circulation (Dixon, 1963), and in which an excess of antibody may lead to an anaphylactic reaction, though failing to remove all circulating antigen. In the chronic case, the large immune complexes are seen as capable of initiating degenerative changes in the liver (and other organs too, perhaps?); while in the persistent carrier, a state of symbiosis and mutual forbearance seems to have been achieved, with neither any obvious reaction against the Au antigen, nor any detectable pathology in the host. These findings will be considered further in the concluding discussion.

F. Immunofluorescence

The use of IF by Millman *et al.* (1969) for the identification of Au antigen in liver biopsy cells from cases of hepatitis has been mentioned above (p. 373). A second paper by the same authors (Coyne *et al.*, 1970) extends and substantiates their earlier findings. They studied 61 patients with a variety of diseases, including 33 in whom there was evidence of viral hepatitis: of these, 24 had Au antigen in the serum; these 24, and a further 6, showed a positive IF reaction for Au antigen in their biopsied liver cells. Of the remaining 28 patients, only 2 had Au antigen in the serum—an inmate of a mental institution and a patient with chronic renal disease who had received many blood transfusions. These, and an additional 7 patients, had IF+ liver biopsies: the diagnoses in-

cluded postnecrotic and biliary cirrhosis, biliary atresia, ? bile duct carcinoma, and hepatic metastasis of a renal carcinoma. The cell smears were prepared by mincing the liver specimens and spreading drops of the resulting suspension on slides, and fixing with acetone. They were stained directly with a fluorescein-labeled rabbit antiserum. Positive IF reactions took 3 forms: discrete particles within the nucleus; diffuse fluorescence of the whole nucleus; and fluorescence of the nuclear rim. Occasionally, particles of fluorescent antigen were also seen in the cytoplasm. Of various other tissues examined, the only positive findings were of a few cells in the bone marrow of 2 hepatitis patients, and of antigen in the liver, spleen, mesentery, and testis of a patient with chronic hepatitis who died of acute myeloid leukemia. Cells of other origin, including buffy coat cells and cells from peripheral lymphocyte cultures, were uniformly negative for Au antigen by IF.

The IF technique would be of particular value in observing the exposure of cells in culture to Au antigen, and following the subsequent events, including the potential synthesis of new Au antigen. Several workers have been attempting such studies, but definitive results are still awaited.

An ingenious IF slide test for Au antigen has been devised by Purcell (1970). Large drops of the blood (or serum) to be tested are spread in a film on glass slides, allowed to dry, fixed with acetone for 10 minutes at room temperature, and air-dried. Unactivated guinea pig sera, with and without anti-Au antibody, are spread over part of the film, allowed to stand in contact for 30 minutes at 37°C, and then removed. The slides are then washed several times with phosphate-buffered saline (PBS), pH 7.4, exposed to fluorescein-labeled horse anti-guinea pig globulin, washed again, and examined with a low-power objective. Specimens containing Au antigen fluoresce with varying intensity, showing numerous bright points of light in the serum phase; the intact formed elements of the blood show no staining. The Au antigen content of sera can be roughly quantitated by testing drops of serial dilutions in the same way.

G. Reversed Passive Hemagglutination

Juji and Yokochi (1969) have made Au antigen-antibody precipitates, then washed and treated them with 3 M NaI to dissociate the anti-Au antibody, which they finally separated and recovered by exclusion chromatography on Sephadex G-200 equilibrated with 3 M NaI. After dialysis against PBS, the antibody was used to coat human group O, Rh-positive red cells which had been prepared by treatment with formalin and tannic acid. Control red cells were coated with normal human γ -globulin. For use as detectors of Au antigen, the antibody-

coated red cells were suspended at 0.5% concentration in PBS, pH 7.2, containing 1% normal rabbit serum.

HA tests, done by the microtiter method, were considered to be more sensitive than ID for detecting Au antigen. Fifty-four assorted sera which were Au+, and 57 viral hepatitis sera which were Au-, by ID were tested by RPHA against both control and anti-Au-coated red cells. Four of the 54 Au+ sera agglutinated the control red cells as readily as the test cells, or more so. With the other 50 Au+ sera, the HA titer against antibody-coated cells was at least 16 times higher. On the other hand, 6 of the 57 Au- hepatitis sera had hemagglutinating titers against the antibody-coated cells which were considered significantly higher (≥ 8 times) than their titers against the control cells.

The method has not yet been tried for the detection of antibody, with the help of cells coated with Au particles or some solubilized fraction thereof. Unfortunately, the antibody-coated erythrocytes have so far proved unstable, and become denatured within a few weeks at 4°C. In theory, this could represent a sensitive and relatively simple way of detecting either antigen or antibody; and perhaps some modification of procedure, e.g., substituting activated Sepharose or another suitable immunoadsorbent vehicle, might provide a more stable test suspension.

H. Immunolectroosmophoresis

The technique of IEOP has been introduced (Prince, 1970; Prince and Burke, 1970; Merrill *et al.*, 1970) in an attempt to increase both the speed and sensitivity of screening potential blood donors for the presence of Au antigen. It involves the forced diffusion of the sera under test into a gel in which the antibody reactant has either been incorporated, or else placed in multiple cups or troughs. The method is therefore as much as 10 times more expensive in standard antiserum than the usual immunodiffusion procedures. However, it can apparently detect ~3 times as many Au+ donors as ID, i.e., up to 4 in every 100 commercial donors, which is estimated to be about 90% of the probable carriers of SH agent in this group (Prince and Burke, 1970). The technique is critically dependent on a number of factors, including temperature, pH, type of buffer, ionic strength, and voltage. But if these can be reliably standardized and controlled, and the tendency to nonspecific precipitation minimized, it may prove to be the method of choice for donor screening, especially since the results are available within a few hours.

I. Radioimmunoassay

In the quest for a method more sensitive than CF in its ability to detect Au antigen, especially in the serum of prospective blood donors,

workers from two laboratories (Walsh *et al.*, 1970; Collier and Millman, 1970) recently reported the successful application of the radioimmunoassay procedures which have already proved useful in the measurement of hormones. These entail the preparation of purified Au antigen, and its radioactive labeling with ^{125}I . The purification of Au antigen from serum was effected by either (1) two equilibrium centrifugations in CsCl, followed by a rate separation in sucrose (Gerin *et al.*, 1969), or (2) enzymatic treatment of Au particles concentrated by spinning, followed by gel filtration on Sephadex G-200, velocity sedimentation in a sucrose gradient, and centrifugation to equilibrium in CsCl (Millman *et al.*, 1970a). After the labeling with radioiodine, Walsh *et al.* refractionated the conjugated Au particles on a Sephadex G-200 column, determining the peaks of radioactivity and immunoreactivity by radioimmunodiffusion. They found that most of the ^{125}I was present in the albumin and iodide fractions, but that all the immunoreactivity (together with $>5\%$ of the original label) emerged in the void volume.

In the preliminary standardization, mixtures containing constant amounts of radioiodinated Au antigen (^{125}I -Au) and standard anti-Au antibody reactant are combined with varying amounts of a selected unlabeled Au-positive serum. The effect of the different concentrations of unlabeled antigen in blocking immune aggregation of the ^{125}I -Au is measured by determining the distribution of radioactive counts between precipitate and supernatant, after these have been separated, e.g., by sedimentation, or by chromatoelectrophoresis (Berson *et al.*, 1956). Walsh *et al.* (1970) used an Au+ serum designated "JM," and assigned it a potency of 10^6 "JM units" of antigen per milliliter. They derived a standard curve by plotting the ratio, B/F, of antibody-bound ^{125}I -Au (B) to free ^{125}I -Au (F) as a function of the concentration of standard Au antigen "JM." This showed a marked change in B/F (from 0.5 to 0.25) over the range of Au antigen concentrations from 0 to 50 JM units/ml; then a reduction in B/F from 0.25 to 0.1 corresponding with the increase in Au from 50 to 200 JM units/ml; and above this antigen concentration, only a gradual decline in the value of B/F. It is evident, therefore, that the method is particularly sensitive in measuring differences in the Au antigen content of unknown specimens when the antigen is present in concentrations equivalent to those in 1/10,000 to 1/100,000 dilutions of the standard JM serum. The titer of this serum by CF was 1/1024, indicating that the radioimmunoassay method is some 10-100 times more sensitive for the detection of antigen than the CF technique used.

Walsh *et al.* separated the antigen-antibody complexes by chromatoelectrophoresis (Berson *et al.*, 1956). To separate all bound ^{125}I -Au, including soluble immune complexes, Collier and Millman (1970) pre-

precipitated the antibody with a rabbit antiserum against human IgG, and measured the amount of radioactive antigen in the precipitate. In this way, sera with CF titers of $\leq 1/32$ were found to have radioimmuno-precipitation equivalence titers of 1/5000 to 1/20,000; and antibody could still be detected at a serum dilution of 1/320,000.

Both groups of workers have shown that the method of RIA can thus measure Au antigen and anti-Au antibody in concentrations not detectable by CF, let alone ID. It can also show the concurrent presence of antigen and antibody in a given serum; in such cases, some binding of the ^{125}I -Au is observed in the control without added anti-Au (Walsh *et al.*, 1970). The method appears to hold great promise as the most sensitive means so far available for the detection and quantitation of exiguous amounts of Au antigen and its antibody.

J. The Question of Multiple Antigenic Specificities Related to Australia Particles

In view of the moderately complex nature of the Au particles, it would hardly be surprising if they were found to carry other antigenic determinant sites, in addition to the seemingly single one that has so far been firmly established. They could be present on the particle surface ("epitopes"), or "masked" and waiting to be revealed by suitable treatment ("hypotopes"). In addition, the polypeptides resulting from particle, and protein, disruption, e.g., by sodium dodecyl sulphate and 2-mercaptoethanol treatment, may be expected to have their own antigenic individualities ("cryptotopes"), when appropriate hyperimmune sera have been prepared against them.

The first findings suggestive of multiple antigenic specificities were obtained by Levene and Blumberg (1969). They hyperimmunized rabbits with 2 different antigen preparations: (1) an Au+ serum—containing "Au(1)"—from a patient with leukemia; and (2) serum from a patient with leukemic reticuloendotheliosis, which had given an "unusual and inconsistent reaction" with the aforementioned anti-Au(1) antiserum. The antiserum prepared against this second human serum was called anti-Au(2). By testing various combinations of antigen-containing sera with these two antibody reactants, the authors concluded that at least 3 distinct epitopes were involved, which they designated *a*, *b*, and *c*. The anti-Au(1) serum identified *a* and *c*; the anti-Au(2), *a* and *b*. They proceeded to test sera from different populations with these two antisera and obtained markedly divergent results, as shown in accompanying Table II.

If the two rabbit antisera were being used at dilutions selected to give roughly comparable concentrations of the unshared antibody specificities

anti-*b* and anti-*c*, then antigenic specificity *b* is present and expressed in serum far more frequently than specificity *c*. However, reactions of specificity *a* would blur the picture. The authors did not absorb their antisera to try to obtain unispecific anti-*b* and anti-*c* reactants. Nor was it determined whether epitope *b*, or *c*, as the case may be, is present together with epitope *a* on the same particle, or whether at least two populations of particles are involved.

Millman *et al.* (1970b) have provided further information concerning the antigenic complexity of the Au particles, as reflected in the antibodies elicited in hyperimmunized animals. It was found that the previously described anti-Au(2) activity (Levene and Blumberg, 1969)

TABLE II
FREQUENCY OF AUSTRALIA ANTIGEN REACTING WITH ANTI-Au(1) AND ANTI-Au(2) RABBIT ANTISERUM IN DIFFERENT GROUPS^a

Population	Total number	Percentage	
		Au (1)	Au (2)
Blood donors	293	0	7.2
Staff of institution for mentally retarded	114	0	52.6
Down's syndrome	209	31.6	74.2
Acute viral hepatitis	58	17.2	70.7
Thalassemia (transfused)	128	7.0	96.9

^a All groups comprise individuals from the area of Philadelphia. Some of the thalassemic patients are from New York City and Northern Italy. Table modified from Levene and Blumberg (1969).

decreased with time on storage in the frozen state, disappeared after the immunized rabbits were reinjected with Au antigen, and, moreover, could not be found in the IgG fraction eluted from DEAE-Sephadex. However, it was apparently not demonstrated directly in the IgM fraction at this point. Ascitic fluids from immunized mice (see above, p. 375) generally showed reactions of identity with human and rabbit antisera, and were found to react with 84 of 111 sera (76%) that gave a positive Au reaction with a human antiserum. Six sera were found which reacted with mouse ascitic fluid, but not with human antiserum. By chromatography on DEAE-Sephadex, and gel filtration on Sephadex G-200, anti-Au activity was demonstrable in the apparent IgM as well as the IgG fractions.

Several such fluids formed two bands of precipitate with certain Au+ human sera, although these same sera gave only one band with other

antibody reactants. The two antigens concerned both migrated as α_2 -globulins. The two antibodies were not removed from one such ascitic fluid by absorption with normal human serum. Treatment of this fluid with mercaptoethanol eliminated one antibody, but not the other. These data are interpreted as additional evidence for multiple specificities among the populations of antibodies reacting with Au particles. However, the separate identity of the antigens has not yet been shown by absorption tests or possible physical fractionation.

A further possible indication of antigenic complexity was found by Millman *et al.* (1970a) in suspensions of Au particles purified by enzyme digestion, gel filtration, and zonal and isopycnic centrifugation. After treatment with ether or chloroform for 1 hour at room temperature, such a suspension gave two precipitate bands with a human and a rabbit anti-Au(1) antiserum, with which it had previously only given one. One of the two bands appeared to be common to both the ether- and the chloroform-treated Au particle concentrate; but the other bands, in each case, were distinct and crossed in a reaction of nonidentity.

Experiments in progress in this laboratory (Le Bouvier, 1969) likewise point to a "dissecting" action by trypsin on Au antigen. Exposure of Au+ serum to 2.5% (w/v) trypsin for 1-2 hours at 37°C caused the progressive degradation of a sensitive component (S), leaving a relatively trypsin-resistant component (R), which reacted with certain human anti-Au sera, but not with a rabbit or a mouse anti-Au(1) antiserum (kindly supplied by Dr. B. S. Blumberg), nor with a guinea pig antiserum (generously provided by Dr. R. H. Purell). The S component would therefore appear to be Au antigen of generally recognized specificity; while the precise nature and relationship of the R component remain to be established.

VI. IMMUNOLOGY OF "AUSTRALIA-POSITIVE" HEPATITIS

A. Introduction

Blumberg and co-workers observed the presence of Au antigen in the serum of patients with hepatitis of presumed viral origin (Blumberg *et al.*, 1967a); and Prince (1968a), and subsequently other workers (see above, Section III), demonstrated the appearance of this antigen late in the incubation period of SH, as distinct from IH, its transient presence in most cases, and its apparently tolerated persistence in some. The following section deals with the problem of the immunological responses elicited by the agent of SH which is responsible for the appearance of Au antigen in the serum. Why is there no consistent and lasting production of high levels of antibody? What is the significance

of the "immune complexes" detected in the acute phase, and again in later stages of the disease? And is there any evidence concerning the stimulation of cell-mediated immunity, and its relationship to the course of the infection?

B. Antibody Responses

The antibody response to Au antigen depends upon the method of stimulation and presumably on the dosage of antigen. In repeatedly transfused patients, relatively small amounts of Au antigen (together with subinfectious concentrations of the active SH agent) are administered at intervals, and result in immunization of the recipient with what is, in effect, an inert lipoprotein antigen preparation. The result is not the usual process of infection with production of Au antigen, but instead the elaboration of anti-Au antibody. In 11 of 12 sera examined (Shulman and Barker, 1969), the antibody resembled IgG in size; while in one serum, half of it was of molecular weight $>200,000$. Conceivably at an early stage of immunization IgM antibody may have been made in every case.

In patients undergoing SH-agent infection, it appears that anti-Au antibody may be—and perhaps regularly is—made in the middle of the incubation period, when it is generally not detectable in free form, but only complexed with antigen so as to produce a specific "anticomplementariness" of the patient's serum (*vide supra*). Shulman and Barker (1969) found this anticomplementary (AC) activity to be usually transient, and to precede the appearance of free Au antigen in the serum, though it could also coexist with, appear after, or occur without detectable free Au antigen. Purcell *et al.* (1969) reported AC activity in 4 of 32 hepatitis patients, which was temporally related to the disease, and declined without being replaced by free Au; while in one patient both AC activity and free Au antigen developed concurrently. Au antigen-antibody complexes would, of course, not be detectable by ID, unless dissociated before testing, as was shown by Millman *et al.* (1970c) (*vide infra*: Section VI,C); but such complexes have been seen in the electron microscope in a case of acute hepatitis (Almeida and Waterson, 1969). In 3 of 22 cases, during the month after their Au antigen declined and disappeared, free anti-Au CF antibody was ephemerally detectable (Shulman and Barker, 1969). In at least one patient, two such "cycles" of free antigen and free antibody succeeded one another over a period of a few weeks (Krugman and Giles, 1970). In general, however, free anti-Au antibody is not evident in SH infections, but only in the hyperimmunized recipients of Au+ blood transfusions. In two recipients of large amounts of blood (17 and 24 units, respec-

tively), in whom no antibody was initially detected, a sharp rise in anti-Au CF antibody took place within 1-2 weeks of transfusion, suggesting a previous subliminal sensitization with Au antigen and a secondary antibody response on restimulation (Holland *et al.*, 1969).

Patients with chronic active hepatitis (CAH) often possess an antibody reacting with smooth muscle (SMA). In view of the differing observations on the presence of Au antigen in CAH, Wright (1970) has looked for Au antigen and SMA in sera from patients with CAH of insidious onset, as well as from patients with acute and prolonged viral hepatitis, and with subacute hepatic necrosis (SHN) which had progressed to cirrhosis. SMA was found in 19 of 78 patients with acute viral hepatitis, both early and late in the disease, and just as often in Au-positive as in Au-negative cases; but it was only present at low titer in 2 of 13 patients with prolonged classic viral hepatitis, 6 of whom possessed Au antigen. In the patients with SHN or CAH, Au antigen and SMA occurred together only in one case of SHN. Among the other 14 SHN patients, Au antigen was present in 3, and SMA in 8; while in the 23 patients with CAH, Au antigen was found in 6 and SMA in 10. There was no evidence that SMA behaves in any way as an antibody against Au antigen. These findings are taken to suggest that there may be at least two etiologically distinct forms of CAH, or that the disease, after being initiated by the SH agent (with production of Au antigen), may be perpetuated by some other mechanism such as an autoimmune process.

C. Formation of Immune Complexes

The presence of circulating Au particle-antibody aggregates in acute viral hepatitis has been mentioned (see above, Section V,E). In the fulminating case described by Almeida and Waterson (1969), the aggregates were comparatively small (e.g. <100 particles, and often <10), and all particles, including unaggregated ones, were seen to be coated with an ample supply of IgG antibody molecules. Here, therefore, the situation appeared to be one of antibody excess. These authors also reported the EM findings on the serum of 2 other cases: a patient with "chronic active hepatitis," with minor recurrent symptoms and raised, fluctuating transaminase levels; and an asymptomatic individual manifesting a persistent, and apparently tolerated, infection with SH agent, resulting in the continuing presence of high levels of Au antigen in the serum. In the former case, some antibody was continuing to be made, and caused the formation of large aggregates of spherical and tubular Au particles, together with some free particles not coated with antibody: the picture was one of antigen excess. In the persistent carrier, the Au

particles were all free, uniformly dispersed, and without any evidence of attached antibody.

Millman *et al.* (1970c) demonstrated the presence of Au antigen-antibody complexes in the serum of two individuals: in one, a case of acute viral hepatitis, only antigen could be detected by ID; in the other, a mentally retarded patient, only antibody. Antibody was separable from "low affinity" complexes by centrifugation, and from "high affinity" complexes by treatment with a series of proteolytic and other enzymes. The suggestion is that Au complexes may be present, at some stage, in all individuals in whom free antigen or antibody is detectable.

D. Cell-Mediated Allergic Responses

The question of cellular immunity in relation to Au antigen has so far received little attention. In earlier studies, Mella and Lang (1967) reported an anergic response to phytohemagglutinin (PHA) in cultured peripheral lymphocytes from each of 12 cases of "acute infectious hepatitis;" it was not stated whether these included any examples of SH. Before the onset of the disease, 8–20% of cells were undergoing mitosis. At the height of the illness, the proportion had fallen to 0–0.5%. It rose again during convalescence to 13–20%, by which time various chromosomal anomalies had developed, including stickiness, multiple breaks, deletions, and additions.

Willems *et al.* (1969) tested the response to PHA of leukocytes from 15 patients with viral hepatitis, both IH and SH. In 8, there was a significant depression of DNA synthesis, as judged by thymidine-³H uptake, in the cells after 3 days in culture in medium containing fetal calf serum; but after 6 days' incubation, there was no difference from the controls. The early hyporesponsiveness was unaltered when sera of healthy donors or hepatitis patients were added to the cultures, suggesting that it was inherent in the cells. It was seen in all samples taken within the first week after onset of jaundice, in both IH and SH cases. There was no correlation, in SH, between the detectability of Au antigen in the serum and the lack of lymphoblastic response to PHA after 3 days in culture. Acute phase sera from two IH and two SH patients inhibited the PHA response of normal leukocyte cultures slightly more than did sera of healthy donors; but this effect could not be serially transmitted to fresh cultures. Recently, Agarwal *et al.* (1970) have also confirmed the diminished response of cultured leukocytes to PHA, measured by reduction of DNA polymerase induction and of thymidine-³H incorporation into DNA, in 7 patients with viral hepatitis of unspecified type.

It seems probable, therefore, that early in SH there does regularly develop a depression of the cell-mediated allergic response; and it

remains to be seen if a similar, but persistent, anergy can be demonstrated in those individuals who go on to become chronic carriers of Au antigen.

No comprehensive comparisons appear to have been made, as yet, to determine the occurrence of an Au-specific, as distinct from a more general, anergy in the different kinds and phases of SH-agent infection. (This topic is discussed in Section VII: see below, p. 393). Although Au particles do not *seem* to act as potent stimulators of humoral immunity, it must be remembered that the enormous concentrations of available circulating antigen (estimated at up to 4–40 $\mu\text{g}/\text{ml}$ of serum) would be adequate to sequester large amounts of antibody. In those cases in which immune aggregates are visible, and in which the concentration of circulating antigen falls relatively soon, it is quite probable that an active cellular allergy has also been elicited. In at least a proportion of the chronic particle carriers, on the other hand, it would appear that the formation of anti-Au antibody has been suppressed, and a state of tolerance induced. Since it is more usual, in states of "split tolerance," to find continued antibody synthesis in the absence of cell-mediated reactivity, it seems likely that in persistent possessors of Au antigen the cellular allergic response is likewise suppressed, and that a situation obtains which is analogous to that of mice persistently and tolerantly infected with lymphocytic choriomeningitis virus (cf. Volkert and Larsen, 1965).

VII. CONCLUSIONS AND SPECULATIONS

There can no longer be any serious doubt that the synthesis of Au antigen, and its appearance in the serum, is specifically associated with infection by the causal agent of the SH type of viral hepatitis. The precise relationship of the Au-antigenic particles to the infectious agent is still not settled. There appear to be 4 main possibilities: (1) the smallest units, or "monads" (see above), i.e., those of ~ 16 nm modal diameter, may represent the virus particles, of which presumably only a minority would possess a full complement of nucleic acid, have a relatively high density, and be infective, while the elongated forms would in fact be aberrant, analogous to those seen, for example, among the papovaviruses; (2) most of the particles would represent incomplete virions, or even fragments of a viral envelope similar to that of the myxo- or herpesviruses, and the infectious unit would consist of a rare, relatively dense, "complete" particle containing a nucleocapsid within the membranous envelope; (3) the infectious entity may not be an "orthodox" type of virion at all, but rather some other kind of self-replicating macromolecule, e.g., a unit of lipoprotein membrane with

associated carbohydrate residues, but devoid of essential nucleic acid, as in one of the forms that have been suggested for the agent of scrapie (Gibbons and Hunter, 1967); (4) the Au-antigenic particles may bear no structural relationship whatever to the SH infectious agent, but may be new or modified cellular components whose synthesis is specified by, or specifically derepressed by, the infecting virus.

At the present time, it is not possible to choose between these different hypotheses. The answers to a variety of questions, some of them hereafter adumbrated, will affect the balance of favor between them. Can a small proportion of particles be found, which do contain significant amounts of ribonucleic and/or deoxyribonucleic acid? If so, is infectivity associated with this fraction rather than with the less dense fractions which comprise the bulk of the Au+ particles? Do various forms of treatment, e.g., heat, ultraviolet irradiation, enzyme digestion, extraction with lipid solvents, exposure to sulfhydryl reagents or periodate, leave such infectivity essentially undiminished? Or is there a significant reduction in titer? Can infectivity (and antigenic reactivity) still be demonstrated in any subparticulate fractions? Can macromolecules of normal liver, or other, cells be detected in the particles possessing Au antigenic reactivity?

What is the significance of the relatively large particles reported by Dane *et al.* (1970) and by others (see above, Section IV, D)? Apart from these, and the usual, smaller Au-antigenic particles, are any other morphological entities discernible in sera from SH patients, e.g., comparable with the coronavirus-like particles that have been seen in the serum of certain cases of Au- chronic active hepatitis (Zuckerman *et al.*, 1970), and also in sera from healthy individuals (Deinhardt *et al.*, 1970)?

Clearly, the essential prerequisite for studies of this kind is a relatively simple and reproducible test of infectivity, either *in vivo* or *in vitro*. It is to be hoped that cultural manipulations of cells from different biopsied tissues, including co-cultivation, and perhaps imposed fusion, with diverse indicator cells and organ explants, and possibly also maintenance as heterotransplants, may permit the Au synthetic activity of the infectious agent to be expressed and recognized. And indeed, as Bang (1969) asks, when commenting on the hypothesis that persistent carriage of Au antigen is genetically determined (Blumberg *et al.*, 1969), is there in man, as in the mouse, a specific *cellular* basis for an inherited resistance or susceptibility to infection—and disease—caused by the hepatitis viruses?

Concerning the pathogenesis of SH, attention has been drawn to the possible role of circulating antigen-antibody aggregates (Almeida and

Waterson, 1969), since these were evident in both the acute and chronic disease, but were not seen in the asymptomatic persistent carrier of Au antigen. If the Au particles are being produced by hepatic, and perhaps also by lymphoreticular cells, the immune complexes might be expected to pass into the pulmonary capillaries and, if not there retained, into the systemic circulation, lodging in peripheral capillaries, e.g., in skin, central nervous system, and especially the renal glomeruli. There appear to be no reports, as yet, of lesions related to possible capillary trapping of aggregates, or of the presence of Au antigen or antibody in such situations, in cases of chronic hepatitis, whether in the active, aggressive type, or in the later stages of the relatively benign, persistent form. But such pathology might well be looked for, following the model of mice persistently infected with LCM virus, where the continuing, or slowly developing, production of antibody results in the formation of virus-antibody complexes and a consequent "late disease" of chronic glomerulonephritis (Hotchin and Collins, 1964; Oldstone and Dixon, 1969).

Probably even more important than circulating antibody as a potential pathogenetic factor in viral hepatitis is the cell-mediated allergic response. It seems clear that in the persistent possessor of serum Au antigen, there must be a rather firmly founded state of tolerance, involving not only the antibody synthetic mechanism, but also presumably the cellular immune mechanisms responsible for delayed-type hypersensitivity and the rejection of antigenically foreign or altered cells. Since it is precisely these chronic carriers of large concentrations of Au particles, lacking any detectable allergic response, who remain free from overt disease, suspicion is directed toward such responses as probable essential participants in the varying degrees of hepatic cell destruction that lead to elevation of serum transaminases, symptoms and signs of anicteric hepatitis, or the full-blown icteric disease.

The following model is therefore put forward to explain the differing manifestations of infection caused by the Au antigen-inducing agent of SH. Around the middle of the incubation period (e.g., some 3-7 weeks after inoculation), the infected cells, in the liver and perhaps elsewhere, begin to synthesize new macromolecules with Au antigenic specificity which are either the virion itself, or virus-associated lipoprotein particles, or antigenically altered cell-membrane fragments. These are released into the circulation, to some extent by disintegration of the more susceptible or heavily infected cells, but principally by shedding or budding from cells which otherwise continue to function relatively normally. As the number of disrupted cells increases, so the serum transaminase levels rise. However, there are at this stage no clinical symptoms; and the

patient remains symptom-free, as long as his allergic responses remain minimal or imperceptible. But in many cases, the first-formed Au antigen has already evoked a low concentration of specific anti-Au antibody, all of which becomes bound to the growing numbers of Au particles. This antibody soon declines, to be followed by an excess of free Au antigen in the serum, which generally falls away, in its turn, within a few days to a few weeks. In some cases, there is then a detectable, usually transient, resurgence of antibody, which now begins to circulate in unbound form. In others, there may be subliminal levels of antibody, too low to be picked up by the methods of detection at present available.

Patients in the earlier phase, with "anticomplementary" immune complexes in their serum, do not yet show symptoms of hepatitis, despite their possession of bound, and sometimes also of free, antibody. It is therefore suggested that actual *clinical disease* is the result of a *cell-mediated allergic response against infected cells* which are in the process of producing Au particles, and whose surface membrane, and possibly also internal membranes, have acquired the specific new antigenic configuration. An active immunological attack on such hepatic, and other, cells would lead to massive cytolysis, with signs and symptoms indicating varying degrees of liver dysfunction. In many cases, this energetic reaction, assisted perhaps by the residual antibody-producing capacity, destroys all virus-harboring cells and terminates the infection. Such is the outcome usually seen in relatively healthy individuals, e.g., the personnel who staff renal hemodialysis units (London *et al.*, 1969a; Turner and White, 1969). In contrast to these are those other individuals, e.g., patients with chronic renal disease undergoing hemodialysis and maybe also treatment with immunosuppressive agents, in whom the allergic response may be depressed to a greater or lesser degree, and who therefore fail to eliminate the infection. These individuals go on to become partially, or in some cases totally, tolerant persistent carriers of the SH agent and of the Au particles which are its hallmark. In those with varying levels of incomplete tolerance, the cellular (and perhaps also humoral) responses may fluctuate; if so, these patients would be expected to show variations in their level of serum Au antigen, and would also experience corresponding exacerbations and remissions of their clinical condition. The more perfect and unbroken the tolerance, the smaller would be the clinical disturbance, and the more continuous and sustained the production of Au antigen. In some of these highly tolerant individuals, the SH agent might then indeed begin to behave as a "slow virus," causing a gradually progressive destruction of one or more kinds of tissue, either by its own replication alone, or with the help of periodic, local, minimal, and soon circumscribed allergic reactions.

The different aspects of this hypothetical model, though still for the most part untested, would seem to be consistent with the known facts; and they do point to several kinds of approach that might be expected to shed some light on the question of pathogenesis, such as studies of delayed-type hypersensitivity reactions and *in vitro* lymphoblast responses in the different clinical categories, and stages, of Au+ SH-agent infection.

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