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SERUM IMMUNOGLOBULIN LEVELS IN AUSTRALIA ANTIGEN POSITIVE AND AUSTRALIA ANTIGEN NEGATIVE HEPATITIS

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SUMMARY

Ig levels were determined by radial immunodiffusion in uncomplicated cases of acute hepatitis with or without Australia antigenaemia. Initial sera from Australia antigen negative cases showed a striking elevation in IgM levels when compared to Australia antigen positive cases (6·5 versus 1·9 mg/ml). None of twenty-four Australia antigen positive cases exceeded 3 mg/ml IgM, and only 3/58 Australia antigen negative cases exhibited values below 3 mg/ml. Initial sera from Australia antigen positive and Australia antigen negative subjects did not differ in concentration of IgG, IgA, or IgD. Serial determinations of IgG revealed a transient fall in patients with Australia antigen positive hepatitis, and a rise in Australia antigen negative cases. Asymptomatic, Australia antigen positive, Guaymi Indian subjects were compared to matched Australia antigen negative controls from the same indigenous group and no differences in the concentration of IgG, IgM, IgA or IgD were found, although elevations of IgG and IgM were common in both groups. No evidence of abnormal proteins was found when sera were tested by cellulose acetate electrophoresis or by immunoelectrophoresis versus immunoglobulin-specific antisera. Ultracentrifugal analysis failed to detect '7S' IgM.

INTRODUCTION

The complex changes in serum protein fractions in acute and chronic liver disease have been widely studied. An increased serum globulin during infectious hepatitis was found in 1948 (Havens & Williams, 1948), and by paper electrophoresis this rise was shown to represent γ-globulin (Osserman & Takatsuki, 1963). Studies of specific Ig fractions in naturally occurring infectious hepatitis cases frequently have demonstrated elevations of IgM as well as rises of IgG (Osserman & Takatsuki, 1963; Tomasi & Tisdale, 1964; Lee, 1965; Hobbs, 1967; LoGrippo, Hayashi & Sharpless, 1967; Bevan, Taswell & Gleich, 1968; Wollheim, 1968; Schmidt & Hofstetter, 1968). Wollheim (1968) and LoGrippo *et al.* (1967)

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noted that the IgM elevations which were so striking in 'infectious hepatitis' were much less pronounced in 'serum hepatitis.' Subsequently, Giles & Krugman (1969) were able to show a high, prolonged rise in IgM levels in thirty mentally retarded children experimentally infected with a strain of short-incubation hepatitis (MS-1) obtained from the Willowbrook Institution. Twenty-eight subjects infected with a long-incubation, serum hepatitis-like, strain (MS-2) showed little change in serum IgM concentration. The short-incubation MS-1 strain was found to cause Au-negative hepatitis while the MS-2 strain produced Au-positive hepatitis (Giles *et al.*, 1969). Although LoGrippo *et al.* (1966) found elevated IgM levels in 60% of actue hepatitis cases sampled during an outbreak in an institution for the mentally retarded, evidence subsequently has been presented in abstract form that at least one-third of the cases were Au-positive and that IgM levels did not correlate with Au status (Hayashi & LoGrippo, 1970). A recent study (Peters & Ashcavia, 1970) of IgM and IgG levels in naturally occurring hepatitis cases failed to show any difference between Au-positive or Au-negative cases, although about two-thirds of the patients had some elevation of IgG and/or IgM. That report did not detail the criteria for diagnosis and gave little data relevant to the stage of disease when most blood specimens were obtained.

We investigated Ig levels of acute hepatitis cases in order to learn if the correlation between IgM elevation and Au-positive hepatitis could be confirmed in naturally occurring hepatitis in the community.

METHODS

Patients were drawn from three hospitals in Panama. Gorgas Hospital in the Canal Zone serves primarily U.S. Armed Services personnel and U.S. and Panamanian employees of the Panama Canal Company. Hospital Seguro Social in Panama City serves a population composed of predominantly urban lower- or middle-class Panamanian workers enrolled in the national social security program. Hospital Santo Tomás, also in Panama City, serves a very heterogeneous Panamanian group generally of a lower economic stratum than Hospital Seguro Social. Patients were sought by twice-weekly visits to these three hospitals. All patients were interviewed and sampled by a trained nurse; two-thirds also were interviewed by one of us (CJP). Charts were reviewed and patients with a typical clinical history and glutamic oxalacetic or pyruvic transaminase values greater than 250 units were accepted as acute cases. About three-fourths of the cases at Hospital Seguro Social had liver biopsies and in all biopsied cases the diagnosis was confirmed histologically. The subjects were divided into five groups as follows:

Group A was composed of consecutive cases of acute hepatitis admitted to Gorgas Hospital and to Hospital Seguro Social. Excluded from this group were patients with underlying disease, a history of parenteral narcotics use, a previous attack of hepatitis or a history of transfusion in the past 6 months. All were bled within 1–5 days of admission. These cases were tested for IgM and included ten Au-positive and thirty-four Au-negative cases from Gorgas Hospital, plus twenty-four Au-positive and twenty-four Au-negative cases from Hospital Seguro Social. Balanced groups of patients were chosen from the above categories to test for IgA (twenty-four subjects), IgD (thirty-five subjects), and IgG (fifty-four subjects) levels.

Group B. In addition, serial IgM (twenty-two subjects) or IgG (twenty-seven subjects) determinations were carried out in representative cases drawn from Group A. IgD levels were determined on convalescent as well as acute sera from fourteen cases.

Group C consisted of six acute cases of Au-positive post-transfusion, hepatitis from Hospital Santo Tomás and one from Gorgas Hospital, as well as four Au-positive cases from Gorgas Hospital which followed parenteral narcotics use.

Group D comprised fifteen subjects with viral hepatitis or drug-induced hepatic necrosis who are presented to illustrate certain findings (Table 2). Initial samples from patients 7 through 10 are included in Group A.

Group E consisted of fifteen asymptomatic Guaymi Indians sampled as part of an epidemiological study of Australia antigenaemia. Each Au-positive subject was matched with an Au-negative Guaymi control of the same sex and same locality, but from a different family. Ages were matched within one year if the subject was under 12, within 2 years if under 20, and within 5 years otherwise.

Ig levels were determined by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) using antibody-in-agar plates and a standard serum obtained from Melpar, Inc. (7700 Arlington Boulevard, Falls Church, Virginia 22046). The working standard for IgG, IgM, and IgA (IgR01) is said by the manufacturer to contain 1·21, 11·0, and 1·45 mg/ml of IgM, IgG, and IgA based on assay versus purified myeloma proteins. The manufacturer also finds this serum to contain 126, 138, and 123 units of IgM, IgG, and IgA when compared to World Health Organization (WHO) reference standard 67/95. In fourteen determinations on fourteen separate plates and on 6 separate days, we found the commercial standard to be equivalent to 114+12 (mean ± 1 standard deviation) units of IgM based on WHO reference standard 67/91 (Rowe, Anderson & Grab, 1970). Using fifty North American blood donors, the manufacturer finds normal values to be 0·9–1·7, 7·7–11·3, 0·8–2·0, and 0·0–0·3 mg/ml for IgM, IgG IgA, and IgD. During the course of the testing, sera were repeated on subsequent days. Paired values were used to calculate the variance which was divided by the mean to yield the coefficient of variation. The average coefficient of variation for IgM and for IgG was 13%. Sera were coded for testing. Au-positive and Au-negative samples were included on each plate.

Antibody-in-agar plates specific for IgM and standard sera were obtained from two other manufacturers: Hyland, Inc., Los Angeles, California 92626, and Behring Diagnostics, 400 Crossways Park Drive, Woodbury, New York 11797. Three Au-negative hepatitis sera (IgM results from Melpar system 3·6, 6·0, and 7·2 mg/ml), WHO standard 67/97, and standards from all three manufacturers were run using plates from each of the three manufacturers on two different occasions. Melpar and Behring Diagnostic reagents yielded similar values for the three hepatitis sera, but Hyland reagents gave values about one-third lower. Cross-comparison of standards showed that the discrepancy arose from a relatively higher nominal value assigned to the Hyland standard by the manufacturer. All three manufacturers' plates yielded similar values for the three hepatitis sera when results were expressed in terms of the WHO standard. All results in this paper are expressed in terms of the Melpar standard.

Au antigen determinations were done by GD (Peters & Johnson, to be published) and by CFT (Purcell *et al.*, 1969). All 'Au-negative' subjects were negative in the GD test and had CFT titre less than 2. Fifty-six subjects were positive by GD and CFT. Five were positive by CFT alone.

Other tests. Some sera (see Results) were also subjected to one or more of the following: Cellulose acetate electrophoresis was performed with Beckman Microzone® equipment and scanned with a Beckman Analytrol® integrating scanner after staining with azocarmine.

Immunoelectrophoresis was performed on 1 × 3-inch slides using a Gelman-LKB apparatus, 0·05-M barbital buffer of pH 8·2, and 1% agarose. Sera were developed with Hyland and Melpar anti-human Ig reagents and with Hyland and Melpar anti-IgM reagents. Ultracentrifugal analysis was performed in a Beckman Model L ultracentrifuge with an SW39 rotor. Five-tenths millilitre of a 1 : 3 dilution of serum was centrifuged at 35,000 rev/min for 18 hr on a 10 to 40% (weight/volume) continuous sucrose gradient. Ten fractions of 0·5 cc were collected by puncturing the bottom of the tube and each fraction was examined by GD for IgM, IgG, IgA, alpha$_2$ macroglobulin, transferrin, and albumin, using antisera from Hyland Laboratories.

Analysis of results. Results for acute Au-positive and Au-negative hepatitis patients (Group A) were examined separately for each immunoglobulin class. The IgG and IgM data were further subdivided into groups from Hospital Seguro Social and Gorgas Hospital. Data were divided into suitable intervals and analysed by the χ^2 test to ascertain whether the results could be described by the normal or log-normal distributions. Of the sixteen categories, fourteen were compatible with both the normal and log-normal distribution. IgG results in Au-negative hepatitis patients from Hospital Seguro Social and Gorgas Hospital combined were not accepted as normally ($P<0·05$) or log normally ($P<0·025$) distributed. Significance tests between group means were performed on the original data and on log-transformed data by the *t*-test with identical results.

Analysis of serial IgG data was complicated since intervals between samples were not uniform. Points from each patient were connected by a straight line and values were interpolated at 10-day intervals. This was done only if the 10-day point was bracketed by measured values in the preceeding and succeeding 10-day interval. Thus the number of measured points was at least one greater than the number of extrapolated points. The changes in IgG were assessed by the Wilcoxon signed-rank test. To construct Fig. 3, the extrapolated serial values for each patient were divided by the initial value and then all patients in each group were averaged.

RESULTS

Group A. These patients with acute uncomplicated hepatitis manifested a very striking difference in IgM levels (Fig. 1 and Table 1). None of the thirty-four Au-positive cases had IgM values greater than 3 mg/ml (mean 1·9 mg/ml). Of fifty-eight Au-negative subjects, fifty-one (89%) had IgM concentrations of 4 mg/ml or above (mean 6·5 mg/ml). Four Au-negative hepatitis patients exceeded 3 mg/ml, but were less than 4 mg/ml; the remaining three Au-negative patients had less than 3 mg/ml IgM. The latter three cases were all typical of acute infectious hepatitis when admitted to the hospital. One was mild, one severe, and the third complicated by persistent fever and later development of Guillain-Barré syndrome. There were no detectable interhospital, age, or sex differences in IgM levels.

IgG levels for Hospital Seguro Social were slightly higher than those for Gorgas Hospital, both in Au-positive and in Au-negative patients ($0·10<P<0·05$). For this reason, Au comparisons also were carried out within each hospital group. There were no significant differences between Au-positive and Au-negative subjects.

Group B. Determinations of IgM on serial sera of Au-negative patients selected from Group A showed a decline from their elevated values over a period of 2 weeks or more (Fig. 2). Some patients were bled sufficiently early in their course to demonstrate stable or

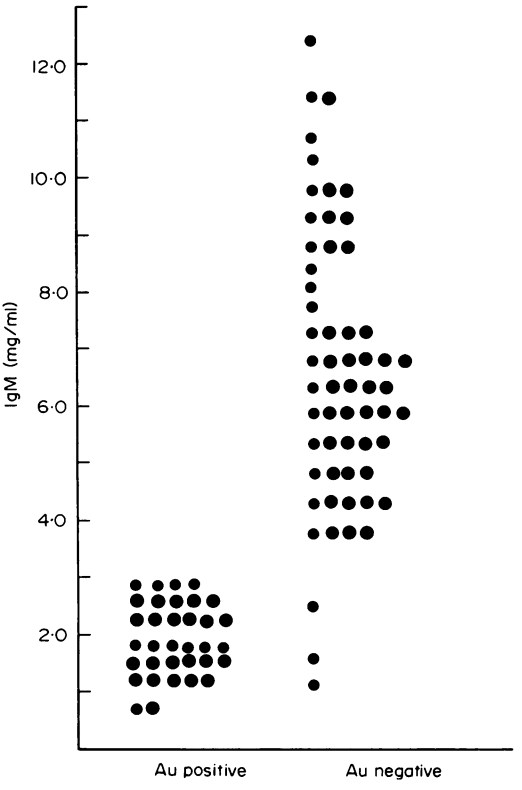

FIG. 1. IgM levels in acute hepatitis.

TABLE 1. Immunoglobulin levels in acute hepatitis

		Au-positive			Au-negative		
		No. cases	Mean	SD*	No. cases	Mean	SD
IgM	GH†	9	1·9	0·8	34	6·9	2·7
	HSS	25	1·9	0·5	24	6·0	2·1
	Both	34	1·9	0·6	58	6·5	2·5
IgG	GH	8	11·9	3·4	18	13·1	5·8
	HSS	17	14·6	2·9	10	16·0	3·5
	Both	25	13·7	3·2	28	14·1	5·2
IgA	Both	10	1·5	0·52	14	1·4	0·51
IgD	Both	15	0·10	0·09	17	0·05	0·03

* $SD = \sqrt{\sum_i (\bar{x} - xi)^2/(N-1)}$.

† GH = Gorgas Hospital; HSS = Hospital Seguro Social.

rising IgM levels. Seven patients with progressively falling IgM levels showed an apparent half-time of 10–34 days (mean 19 days). The serial testing of Au-positive patients showed no consistent change in IgM levels (Fig. 2).

Levels of IgG (Fig. 3) in Au-negative cases increased (six cases) or did not change (four cases). The 10-day average was 17% greater than the day 0 average ($P<0{\cdot}02$). Four Au-positive subjects treated with steroids showed a somewhat more marked decrease. These

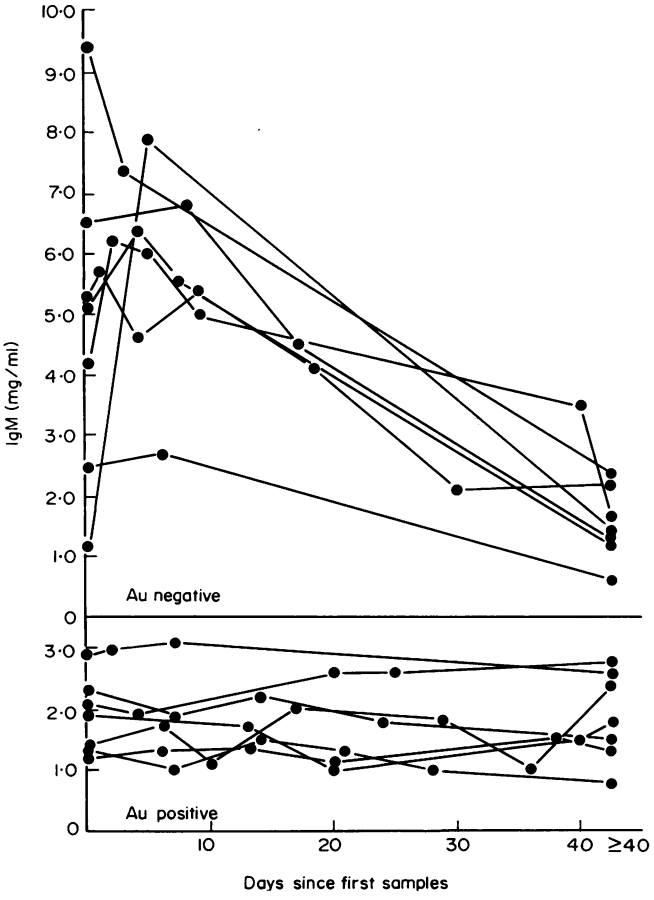

FIG. 2. Serial IgM levels in hepatitis patients.

observations were confirmed by repeating serial sera from each patient on the same plate and at the same dilution. Paired IgD determinations disclosed no significant alteration during the course of Au-positive or Au-negative hepatitis.

Group C. This mixture of Au-positive, post-transfusion and narcotics-associated cases from three different hospitals showed higher levels of IgM (mean 2·9, standard deviation 1·0, range 1·0–4·7 mg/ml, $P<0{\cdot}05$) than the cases in Group A. IgG was also somewhat higher, but not significantly so (mean 15·8, standard deviation 6·6 mg/ml).

Group D. These results are summarized in Table 2. The two patients (1 and 2) who presented with Au antibody had IgM levels of 6·7 and 2·7 mg/ml, higher than those typically seen in hepatitis associated with Au antigenaemia.

Patients 3 through 6 had acute Au-negative hepatitis and had been hospitalized for hepatitis 2–6 years previously. Charts from previous and recent hospitalizations were reviewed, and the clinical and biochemical courses were typical (including negative heterophile antibody determinations). Case 5 exemplifies what one might expect from a subject with Au-negative hepatitis and previous attacks of hepatitis. She was Au-negative (CFT) and IgM was elevated to 6·3 mg/ml. Au antibody was detectable by radioimmunoprecipitation in acute and convalescent sera, suggesting that her first bout of hepatitis was Au positive.

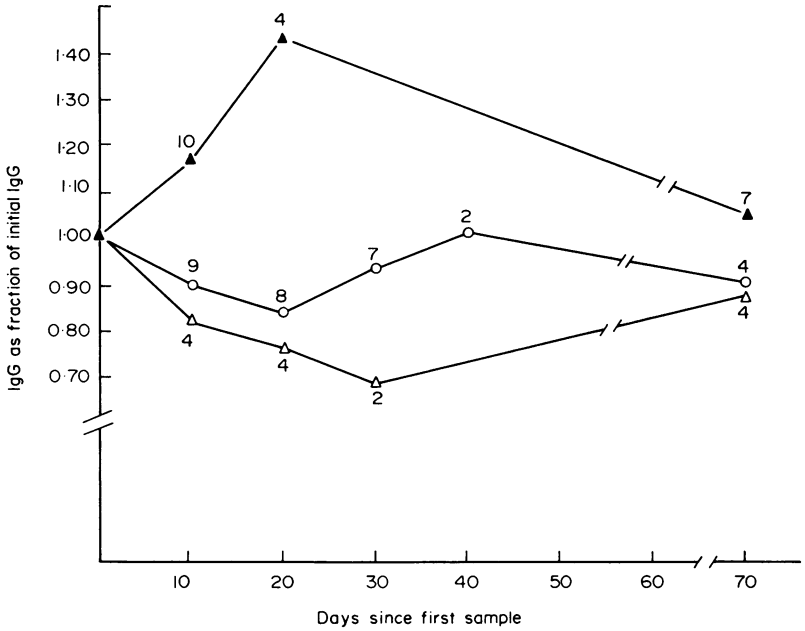

FIG. 3. Serial IgG levels in hepatitis patients. ▲, Au negative; ○, Au positive; △, Au positive with steroids. Arabic numeral indicates patients tested.

Patients 3 and 4, however, had normal IgM levels in serial specimens and neither demonstrated Au binding when acute and convalescent sera were tested by radioimmunoprecipitation. Subject 6 had a history of jaundice with a positive absorbed heterophile, as well as clinical and biochemical evidence of hepatitis following duty in Vietnam. Au antigen was not detected in serial serum specimens and he had an elevated IgM; however, Au antibody was not detected by radioimmunoprecipitation, suggesting that his other attack of hepatitis was not associated with Au antigen.

Subjects 7, 8, and 9 all were initially Au negative, but then relapsed outside the hospital and were readmitted. They showed a decline of their IgM levels towards normal despite clinical and laboratory evidence of worsening of their disease process. Subject 10 was an Au-positive hepatitis patient who initially improved at home, but who suffered a relapse and was hospitalized. He showed no rise in IgM at the time of relapse. Subject 11 presented with

acute Au-positive hepatitis 60 days after his wife fell ill with Au-positive hepatitis. Biopsy revealed chronic active hepatitis, and he showed a progressive rise in IgM levels until his death 7 months later.

Drug-induced hepatitis was considered probable or possible in subjects 12–16. The IgM levels were all relatively low except in case 14.

TABLE 2. Au antigen and immunoglobulin results in selected patients

Patient No.	Reason presented	Days since onset	Au*	IgM	IgG	Anti-Au (RIP)†
1	Au antibody in initial specimen	17	ab	6·7	15·0	+
2	Au antibody in initial specimen	9	ab	2·7	17·6	+
3	Second attack	7	0	1·4	18·2	0
4	Second attack	12	0	1·9	27·4	0
5	Second attack	8	0	6·3	—	+
6	Second attack	12	0	8·8	14·8	0
7	Acute hepatitis with relapse	10	0	5·1	19·7	—
		71	0	2·1	15·9	—
8	Acute hepatitis with relapse	9	0	4·3	—	—
		36	0	3·2	—	—
9	Acute hepatitis with relapse	10	0	6·2	18·7	—
		103	0	2·1	23·5	—
10	Acute hepatitis with relapse	15	ag	1·6	8·1	—
		47	ag	1·8	9·2	—
11	Acute hepatitis progressing to chronic active hepatitis and death	10	ag	2·7	19·4	—
		80	ag	3·9	14·0	—
		192	ag	4·4	19·4	—
12	Acute hepatitis versus methyldopa reaction	10	0	1·4	20·8	—
13	Acute hepatitis versus isoniazid reaction	21	0	2·8	—	—
14	Acute hepatitis versus stelazine reaction	9	0	4·5	—	—
15	Acute hepatitis versus disulfiram or chlordiazepoxide reaction	15	0	1·0	—	—
16	Acute hepatitis versus methyldopa	23	0	2·0	15·6	—

* ab: Anti-Au antibody detected by gel diffusion and complement fixation. ag: Au antigen detectable by gel diffusion or complement fixation. 0: no antigen or anti-Au antibody detectable by gel diffusion and complement fixation.

† Results of radioimmunoprecipitation test for anti-Au antibody performed by Dr R. Purcell and Dr J. Lander.

Group E. The Guaymi Indian sera taken from a region of high incidence of asymptomatic Au antigenaemia showed no difference in IgG, IgM, IgA, or IgD levels when Au positives were compared to matched Au negatives by the paired *t*-test (Table 3). The levels of IgG were elevated in comparison to the hepatitis patients tested here or the manufacturers' results on North American blood donors. The IgM levels were also quite high, although not so elevated as in acute cases of Au-negative hepatitis.

Additional Studies. Sera from thirteen acute Au-positive and twenty-one acute Au-negative hepatitis cases and from ten Guaymi Indians were examined by cellulose-acetate

electrophoresis. No evidence of monoclonal proteins was found. The polyclonal nature of the increased IgM levels in Au-negative hepatitis was further confirmed in five subjects by comparing scans of acute sera to scans of late convalescent sera. Five acute Au-positive and twelve acute Au-negative hepatitis patients had immunoelectrophoretic patterns which were consistent with their quantitative Ig levels when developed with polyvalent Ig or specific IgM antisera. There was no evidence of qualitative abnormality. Acute sera from five Au-negative hepatitis patients (IgM = 11·4, 10·4, 9·2, 8·3, 6·0 mg/ml) and a normal serum pool were analysed in the ultracentrifuge. IgM was detected only in the bottom half of the tube in the same location as alpha$_2$ macroglobulin. Transferrin and albumin were in the upper fractions. IgG was in the upper half of the tube except in three hepatitis patients who had rheumatoid factor, which resulted in the appearance of IgG in the IgM fractions also. IgA appeared with the lower IgG fractions.

TABLE 3. Immunoglobulin levels of Guaymi Indians: Australia antigen carriers and matched controls

Ig	No. of pairs	Australia antigenaemia mean	SD*	Control mean	SD*	Mean difference	SD† of differences
IgM	15	2·7	1·2	2·5	0·8	0·21	1·6
IgG	15	16·9	4·7	15·2	4·2	1·6	5·5
IgA	10	1·3	0·6	1·2	0·6	0·03	0·6
IgD	10	0·1	0·09	0·05	0·04	0·05	0·1

$$* \text{SD} = \sqrt{\frac{\Sigma(\bar{x}-xi)^2}{N-1}}.$$

$$\dagger \text{ SD of differences} = \sqrt{\frac{\Sigma\overline{(x'-x'')-(x'-x'')\,i}}{N-1}}.$$

DISCUSSION

Our results indicate that acute, uncomplicated, Au-negative hepatitis is associated with a substantial rise in the serum concentration of IgM. The protein was shown to be polyclonal and we were unable to detect slowly sedimenting ('7S') components (Solomon & McLaughlin, 1970). Au-positive cases rarely exhibited increased levels of IgM, even when serial samples were tested until Au antigen disappeared from the patient's serum.

The measurement of IgM levels in Au-negative hepatitis should be useful in epidemiological and clinical work as a 'marker' to reinforce the inability to demonstrate Au antigenaemia. The differences between Au-positive and Au-negative cases will be maximal if patients are studied during the first 2 weeks of illness and if other causes of IgM elevation are not operative in the population. The higher IgM levels observed in parenterally transmitted Au-positive hepatitis may have been due to the route of inoculation, but also may have been influenced by intercurrent disease. Our data on cases 12–16, plus the observations of others on chlorpromazine- and methyltestosterone-induced jaundice (Hobbs, 1967; Wollhein, 1968), suggest that IgM levels interpreted in conjunction with Au antigen testing

will also be useful in attempting to decide whether a subject has drug-induced hepatic necrosis or infectious hepatitis. Relapses of Au-negative hepatitis were not associated with an IgM rise, suggesting that they may have a different pathogenesis than the primary attack. It is of interest that a case of acute Au-positive hepatitis that developed chronic active hepatitis showed a progressive increase in serum IgM concentration. The low levels of IgM found in three of four Au-negative patients with a second attack of hepatitis are not explained. It must be borne in mind that more sensitive tests might detect Au antigen in these and other subjects or that specific virological tests could change the entire picture presented here.

The transient fall in IgG in Au-positive hepatitis contrasts with the rise seen in Au-negative hepatitis. These differences are not sufficiently great to be detected in comparing initial IgG values in the two types of hepatitis, but do become apparent on testing serial specimens. It is not clear whether this decrease in IgG is related to the failure to find the marked rise in IgM seen in Au-negative cases. One speculation is that the decreased IgG reflects a process of immunosuppression that may be related to the evolution of chronic antigenaemia observed in some patients with Au-positive hepatitis.

Our studies with the Guaymi Indians were undertaken to investigate the effects of chronic antigenaemia on serum Ig levels. The failure to find a significant difference in Au-positive and Au-negative subjects should not be interpreted as negating the possibility of such differences. The high levels of IgM and IgG observed in 'healthy', subjectively afebrile, Au-negative Indians suggests that this is not the ideal population in which to search for such differences.

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ABBREVIATIONS

Au Australia antigen
GD gel diffusion