Detection of Australia antigen in liver biopsies by immunofluorescence

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Summary: A search for Australia antigen (AuAg) was made by immunofluorescence in 105 liver biopsies obtained from the same number of patients. No specific fluorescence was observed in 38 cases of acute viral hepatitis (19 of them seropositive for AuAg) or in 55 seronegative patients with various liver disorders or in 8 seronegative patients with histologically normal livers. However, specific fluorescence was seen in two cases: in the single case of chronic aggressive hepatitis seropositive for AuAg and in one of three cases of chronic persistent hepatitis with AuAg-positive sera. The fluorescence observed was mainly intranuclear when cellular suspensions were used, but cytoplasmic fluorescence was more prominent when observations were made on cryostat sections. The finding of AuAg by immunofluorescence in liver cells in chronic but not in acute forms of hepatitis seropositive for AuAg is consistent with the hypothesis of an important role of cellular immunity directed against infected cells in the pathogenesis of viral hepatitis.

Résumé: *Détection de l'antigène Australie par immunofluorescence dans les biopsies hépatiques*

L'antigène Australie (AgAu) a été recherché par immunofluorescence dans 105 biopsies hépatiques provenant de patients différents. Aucune fluorescence spécifique n'a été observée dans 38 cas d'hépatite virale aiguë (dont 19 à AgAu positif), ni chez 55 patients à AgAu négatif avec diverses pathologies hépatiques, ni chez 8 patients à AgAu négatif avec un foie histologiquement normal. Une fluorescence spécifique pour l'AgAu a été observée dans deux cas: dans le seul cas d'hépatite chronique agressive à AgAu positif de notre série et dans un cas sur trois d'hépatite chronique persistante à AgAu positif. La fluorescence était localisée principalement dans le noyau quand les observations étaient faites sur des suspensions cellulaires, alors que sur les coupes au cryostat la fluorescence était à prédominance cytoplasmique. La découverte de l'AgAu par immunofluorescence dans les cellules hépatiques dans les formes chroniques, mais non dans les formes aiguës de l'hépatite à AgAu positif est compatible avec l'hypothèse d'un rôle important de l'immunité cellulaire dans la pathogénèse de l'hépatite virale.

In the first studies on the localization of Australia antigen (AuAg) by immunofluorescence, AuAg was found in the nuclei of liver cells of all cases of viral hepatitis seropositive for AuAg as well as of a number of seronegative cases.[1,2] Subsequent studies yielded conflicting results. Some workers found no correlation between the presence of AuAg in the serum and immunofluorescence in hepatocytes,[3] while others reported such a correlation with predominant localization of AuAg in the cytoplasm[4,5] or in both the nucleus and cytoplasm.[6]

In the present study we sought to detect and localize by immunofluorescence AuAg in the cells of liver biopsies taken for diagnostic purposes.

Material and methods

One hundred and five liver biopsies from as many patients were studied. In all cases percutaneous liver biopsy had been performed for diagnostic purposes. A portion of the biopsy was routinely processed for standard histologic examination and a 4- to 6-mm. segment was snap-frozen and stored immediately at -70°C. for immunofluorescence studies. A serum sample taken on the day of the biopsy was tested for AuAg by counter-immunoelectrophoresis.[7] Of the 105 biopsies 97 showed histologic abnormalities while 8 appeared histologically normal. The histological diagnosis and results of the serum determination for AuAg are given in Table I.

For direct immunofluorescence, a human serum containing antibodies to AuAg was treated with 50% ammonium sulfate to form a precipitate and used after labelling with fluorescein isothiocyanate by the method of Clark and Shepard.[8] A normal human serum containing no antibody to AuAg was prepared in an

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identical manner and served as a control.

For indirect immunofluorescence, human antiserum (as above) and rabbit unconjugated antisera against AuAg (Behring Diagnostics) were used in the first step, followed by application of fluorescein-conjugated goat antiserum against human IgG (Meloy Laboratories or Hyland Laboratories) or against rabbit immunoglobulins (Meloy Laboratories). Normal human and rabbit unconjugated sera were used as controls in the first step.

Free fluorescein was removed by passage through a Sephadex G-25 column. All sera were absorbed twice with acetone-dried rabbit liver powder (Pentex). The specificity of the antisera against AuAg was ascertained in immunodiffusion by comparison with an anti-AuAg human serum (kindly supplied by Dr. A. M Prince).

Cell suspensions were prepared from all tissue specimens for immunofluorescent examination by the technique of Millman *et al.*[1] Four specimens were also examined using cryostat sections of 3-μ thickness prepared at -20°C. Tissues were fixed in acetone at room temperature for one or ten minutes, or both, and air-dried.

For the direct fluorescence technique the slides were incubated for 45 minutes at 37°C. in a humid chamber with the conjugated human antiserum against AuAg, then washed in three separate phosphate-buffered saline baths, in the first for 15 minutes and in the second and third for five minutes each. For the indirect fluorescence technique the slides were incubated for 45 minutes at 37°C. either with human or rabbit antiserum against AuAg, then washed as above, incubated again, under the same conditions, with fluorescein-conjugated goat antiserum against human IgG or rabbit immunoglobulin, and finally washed again. The slides were mounted in buffered glycerol and read with a Leitz fluorescence microscope without knowledge of the clinical or histological diagnosis.

In each case careful controls were used. For the direct technique, at least one slide was left untreated with any fluorescein-conjugated serum to assay the primary fluorescence of the tissue, and at least one slide was treated with fluorescein-conjugated normal human serum to assay the secondary non-specific fluorescence. For the indirect technique, 11 slides were prepared in each case, 9 of which were controls, as shown in Table II. A result was considered positive if the following criteria were fulfilled: (1) the fluorescence observed was not found in control slides; (2) the results could be reproduced; (3) the accuracy of the reading could be ascertained by a second operator on coded slides; (4) the fluorescence considered specific could be inhibited with an unlabelled antiserum specific for AuAg.

Table I
Histological diagnosis and serum determination of AuAg

Histological diagnosis	Number of cases	Number seropositive for AuAg
Acute hepatitis	38	19
Chronic persistent hepatitis	3	3
Chronic aggressive hepatitis	2	1
Other hepatopathies*	54	0
Histologically normal liver	8	0
Total	105	23

*In this group the diagnoses were: alcoholic hepatitis (9), micronodular cirrhosis (14), alcoholic hepatitis with cirrhosis (10), metastases to liver (4), non-specific focal hepatitis (4), hepatic steatosis (3) and miscellaneous liver diseases other than the foregoing(10).

Results

Non-specific fluorescence, whether cytoplasmic or nuclear, was found to be a major problem in our study. Cytoplasmic fluorescence was predominantly diffuse while nuclear fluorescence was granular, either coarse or fine and diffuse. Retrospectively, it appeared that non-specific fluorescence was seen mainly in tissues where hepatic cell necrosis or fatty infiltration was one of the histological findings. Therefore adequate controls were absolutely essential.

With the use of proper controls no specific fluorescence was observed in 38 cases of acute viral hepatitis (19 being seropositive for AuAg) or in 54 seronegative patients with various liver disorders or in 8 seronegative patients with histologically normal livers. However, specific fluorescence was seen in two cases: in the only AuAg-seropositive case of chronic aggressive hepatitis (another seronegative case was negative for fluorescence) and in one of the three seropositive cases of chronic persistent hepatitis.

Both positive cases were studied by direct and indirect immunofluorescence techniques and using both cellular suspensions and cryostat sections. The fluorescence observed by either the direct or the indirect technique was essentially the same. However, the fluorescence pattern was different depending upon whether cellular suspensions or cryostat sections were used. With the cellular suspension technique, a predominantly granular nuclear fluorescence was observed as illustrated in Fig. 1; these granules were seen in approximately half of the hepatocytes of the two positive cases of our series. In cryostat sections, a diffuse

Table II
Slides prepared for the indirect fluorescence technique

Slide no.	First incubation	Second incubation
1	nothing added	nothing added
2	nothing added	conjugated anti-human IgG
3	nothing added	conjugated anti-rabbit Ig
4	unconjugated human anti-AuAg	conjugated anti-human IgG
5	unconjugated human anti-AuAg	conjugated anti-rabbit Ig
6	unconjugated normal human serum	conjugated anti-human IgG
7	unconjugated normal human serum	conjugated anti-rabbit Ig
8	unconjugated rabbit anti-AuAg	conjugated anti-human IgG
9	unconjugated rabbit anti-AuAg	conjugated anti-rabbit Ig
10	unconjugated normal rabbit serum	conjugated anti-human IgG
11	unconjugated normal rabbit serum	conjugated anti-rabbit Ig

cytoplasmic fluorescence was more prominent (Fig. 2 — same case as in Fig. 1).

The positive results were verified by the direct technique, using two fluorescein-labelled antisera against AuAg of human and goat origin (kindly provided by Dr. I. Millman). The specificity of the fluorescence observed in these two cases was further ascertained by inhibiting the reaction with unlabelled antiserum against AuAg. No difference in the intensity of the fluorescence was observed whether the slides were fixed in acetone for one or ten minutes.

Discussion

Fluorescence specific for AuAg was seen in liver biopsies of two patients suffering from chronic liver disease with circulating AuAg. However, no specific fluorescence was detected in liver biopsies obtained from two other patients with chronic persistent hepatitis or from 19 patients with acute viral hepatitis, all of whom were seropositive for AuAg. No specific fluorescence was seen in any seronegative case.

Coyne *et al*[2] have found AuAg by immunofluorescence in all biopsies obtained from 26 patients with antigenemia they have examined and in 15 cases where no antigen was detectable in the serum. Edgington and Ritt[4] found specific fluorescence for AuAg in about one third of liver biopsies from patients with acute hepatitis, whether AuAg was detected or not in the serum; similar results were reported by Nielsen and Elling[3] in about two thirds of their biopsies from cases of acute hepatitis. These results are at variance with our own and those of other investigators.[5,6,9] This discrepancy can be attributed to technical factors, to the use of different reagents or to differences in the interpretation of the results. Since one of us has studied seven biopsies in one of the precited laboratories, employing the technique and the reagents of that laboratory, and could not reach agreement on the interpretation of the results, we therefore believe that one of the critical factors is the interpretation which, in our view, can be accurate only with the use of appropriate controls.

Nowoslawski *et al*[6] have studied 12 cases of lymphoproliferative disorders which came to autopsy; six were seropositive for AuAg, the others were seronegative. AuAg was found by immunofluorescence in livers of only the seropositive cases. It is noteworthy that the positive cases reported in this study are of chronic carriers of AuAg. Moreover, Nowoslawski[10] observed that when many specimens from different regions of the same liver were studied, both positive and negative results could be obtained from the same organ. This might well explain why two of our cases of chronic persistent hepatitis were found to be negative for fluorescence on the basis of the study of a fragment of a single biopsy.

More recently, Hadziyannis *et al*[5] reported that they detected AuAg by immunofluorescence in liver specimens from nine AuAg-seropositive patients with chronic liver diseases, from 22 healthy seronegative controls and from 11 seronegative patients with various liver disorders. It is interesting that in their discussion the authors reported initial failure to demonstrate AuAg in the livers of patients with hepatitis. While they ascribed this initial failure to technical difficulties, it might well be, in the light of our results, that AuAg is more easily demonstrated in the livers of chronic carriers than of patients with acute hepatitis. Moreover, this interpretation is in agreement with a recent report by Krawczyński *et al*[9] who could not find AuAg by immunofluorescence in livers removed at autopsy from nine cases of acute or fulminant hepatitis, but obtained positive results in 16 of the 40 patients who died with a more chronic form of the disease.

It is noteworthy that in the present paper and in the three papers just mentioned,[5,6,9] specific fluorescence for AuAg in the liver has been found exclusively in healthy carriers or in patients with chronic forms of

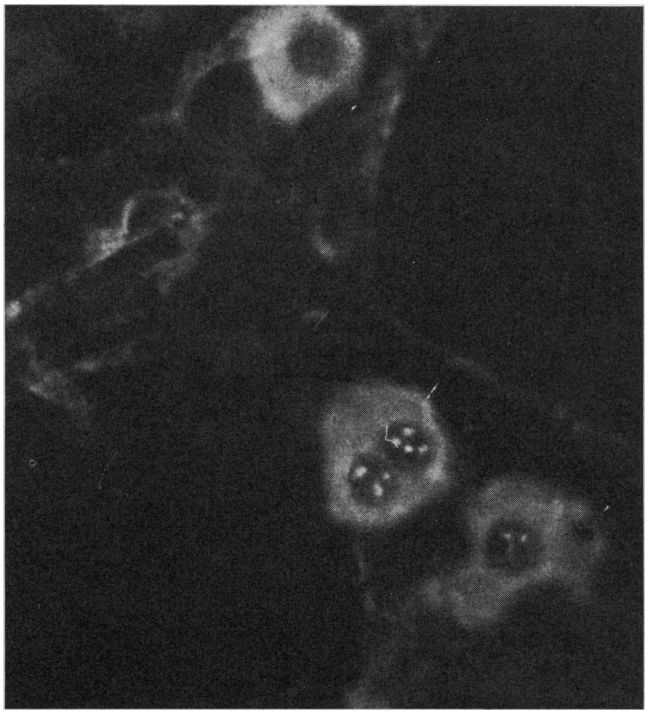

FIG. 1—Nuclear fluorescence in a case of chronic hepatitis: cellular suspension. Original magnification x 400.

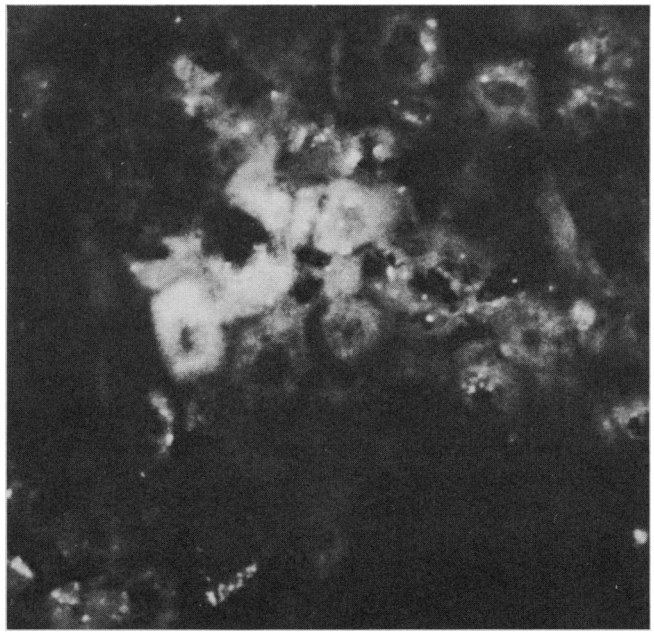

FIG. 2—Predominantly cytoplasmic fluorescence in a case of chronic hepatitis (same case as in Fig. 1): cryostat section. Original magnification x 400.

hepatitis. It must also be pointed out that in electronmicroscopic studies particles resembling AuAg have been found only very rarely[11,12] or not at all[13] in acute forms of hepatitis, whereas they are more commonly found in healthy carriers of AuAg[13,14] or in chronic forms of hepatitis.[9,15] Our results, as well as those just mentioned, are consistent with the hypothesis of Dudley, Fox and Sherlock[16] that acute hepatitis is caused by the cellular immune response of the host against liver cells infected with the hepatitis B virus. In that case the infected cells would be destroyed, explaining why one cannot detect them by immunofluorescence techniques. On the other hand, in patients with insufficient immune response not all infected liver cells would be destroyed, in which case the demonstration of AuAg-infected cells would be possible more or less consistently. Finally, persons who failed to respond to the virus would be healthy carriers and show prominent liver cell involvement by AuAg. This last view could explain the differences in the intensity of the fluorescence observed by Hadziyannis *et al*[5] between chronic and healthy carriers.

The fluorescence observed in liver cells by the use of Au antibody has been described as being either nuclear[1-3] or cytoplasmic[4,5] or both.[6,9] Our results show that this difference might be explained by the technique used for cell preparation. Indeed, when cellular suspensions were used, the fluorescence was almost exclusively nuclear, while when cryostat sections of the same specimens were examined cytoplasmic localization became prominent. Electronmicroscopic studies have shown particles resembling AuAg either in the nucleus[9,11,12] or the cytoplasm,[14] but the recent use of the immunoferritin technique has shown that AuAg can be found in both sites.[15] The conflicting results reported in the literature concerning the subcellular localization of AuAg might well be due to the difference in the techniques of detection.

In conclusion, the present data are in agreement with other reports and suggest that AuAg cannot be found by immunofluorescence techniques in hepatocytes of patients with AuAg-seropositive acute hepatitis but can be identified in the livers of seropositive patients with more chronic forms of the disease or in healthy carriers. These data are consistent with the hypothesis that AuAg-positive acute or chronic hepatitis may be an expression of the cellular immune response of the host against liver cells infected with hepatitis B virus.

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References

1. MILLMAN I, ZAVATONE V, GERSTLEY BJS, et al: Australia antigen detected in the nuclei of liver cells of patients with viral hepatitis by the fluorescent antibody technique. *Nature (Lond)* 222: 181, 1969
2. COYNE VE, MILLMAN I, CERDA J, et al: The localization of Australia antigen by immunofluorescence. *J Exp Med* 131: 307, 1970
3. NIELSEN JO, ELLING P: Investigations of liver biopsies for Australia antigen by immunofluorescent technique. *Clin Exp Immunol* 9: 699, 1971
4. EDGINGTON TS, RITT DJ: Intrahepatic expression of serum hepatitis virus-associated antigens. *J Exp Med* 134: 871, 1971
5. HADZIYANNIS S, VISSOULIS C, MOUSSOUROS A, et al: Cytoplasmic localization of Australia antigen in the liver. *Lancet* I: 976, 1972
6. NOWOSLAWSKI A, BRZOSKO WJ, MADALIŃSKI K, et al: Cellular localisation of Australia antigen in the liver of patients with lymphoproliferative disorders. *Lancet* I: 494, 1970
7. MAILLOUX-CÉRAT L, TURGEON P, ROBERT J, et al: L'antigène Australie dans l'hépatite: aspects techniques et cliniques. *Union Med Can* 100: 2203, 1971
8. CLARK HF, SHEPARD CC: A dialysis technique for preparing fluorescent antibody. *Virology* 20: 642, 1963
9. KRAWCZYŃSKI K, NAZAREWICZ T, BRZOSKO WJ, et al: Cellular localization of hepatitis-associated antigen in livers of patients with different forms of hepatitis. *J Infect Dis* 126: 372, 1972
10. NOWOSLAWSKI A: Personal communication
11. SCOTTO J, STRALIN H, CAROLI J: Amas de particules viriformes dans les noyaux d'hépatocytes chez un sujet atteint d'hépatite virale mortelle et porteur d'antigène Australie dans le sérum. *C R Acad Sci [D] (Paris)* 271: 1603, 1970
12. NELSON JM, BARKER LF, DANOVITCH SH: Intranuclear aggregates in the liver of a patient with serum hepatitis. *Lancet* II: 773, 1970
13. CARAMIA F, DE BAC C, RICCI G: Virus-like particles within hepatocytes of Australia antigen carriers. *Am J Dis Child* 123: 309, 1972
14. STEIN O, FAINARU M, STEIN Y: Virus-like particles in cytoplasm of liver of Australia antigen carriers. *Lancet* II: 90, 1971
15. HUANG SN, MILLMAN I, O'CONNELL A, et al: Virus-like particles in Australia antigen-associated hepatitis. An immunoelectron microscopic study of human liver. *Am J Pathol* 67: 453, 1972
16. DUDLEY FJ, FOX RA, SHERLOCK S: Cellular immunity and hepatitis-associated Australia antigen liver disease. *Lancet* I: 723, 1972