Australia Antigen and Antibody in Transfused Children with Thalassaemia*

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Vierucci, A., London, W. T., Blumberg, B. S., Sutnick, A. I., and Ragazzini (1972). *Archives of Disease in Childhood*, 47, 760. **Australia antigen and antibody in transfused children with thalassaemia.** As a consequence of frequent transfusions, 10% of 169 Italian patients with thalassaemia developed Au(1) and 20% anti-Au(1). Au(1) persisted in the children in whom it was detected for the duration of the study (2–7 years) or until the patient died. Anti-Au(1) was less persistent.

In these children, Au(1) or anti-Au(1) was detected, but not both, suggesting that patients with persistent Au(1) and antibody formers represent two distinct subgroups of the thalassaemia population. Au(1) was more common in males and in patients less than 7 years old, and was associated with earlier death; whereas antibody was more common in females, and in children older than 7 years, and was associated with longer survival.

Australia antigen was discovered as a result of a systematic search for human serum protein polymorphisms (Blumberg, 1964). Epidemiological, morphological, biochemical, and tissue culture data strongly support the hypothesis that Australia antigen Au(1) is (or is located on) an infectious agent which can cause hepatitis in man (Sutnick, London, and Blumberg, 1967; Blumberg, Sutnick, and London, 1970c; Shulman, Hirschman, and Barker, 1970; Gerin *et al.*, 1969).

In acute viral hepatitis Au(1) appears early in the disease, often before any clinical signs or symptoms, lasts a few days or weeks, and then becomes undetectable. Some people, however, are chronic carriers of Au(1). They have little or no evidence of liver disease but transfusion of their blood can transmit hepatitis to recipients. Studies have shown that 1 to 20% of apparently normal persons among certain (mostly tropical) populations have Au(1) in their blood (Blumberg *et al.*, 1970c). Family studies by our group and in Ceppellini's laboratory (Ceppellini *et al.*, 1970) are compatible with the hypothesis that suscepti-

bility to become an Au(1) carrier is inherited as an autosomal recessive trait.

On systematic follow-up studies of 189 recipients of Au(1) blood, 131 developed hepatitis (as denoted by serum glutamic pyruvic transaminase (SGPT) elevation), 65 developed Australia antigen, and 29 antibody to Australia antigen. Of 345 recipients of only Australia antigen negative blood, 93 developed SGPT elevation, 14 developed Au(1), and 2 anti-Au(1). Some of the people who developed Au(1) or anti-Au(1) had no apparent clinical illness. Thus, the recipient of blood containing Au(1) is likely to develop hepatitis, Au(1), or anti-Au(1) (Goesser *et al.*, 1970; Okochi *et al.*, 1970; Gocke, Greenberg, and Kavey, 1970; Goldfield, 1970).

Anti-Au(1) antibodies (detected by immunodiffusion) have been found primarily in sera from transfused patients with haemophilia, aplastic anaemia, and thalassaemia. They have also been detected in patients who have never been transfused; some of these have had clinical hepatitis, but others have had no apparent illness. The observations are consistent with the hypothesis that antibodies form in patients 'infected' with Au(1) either by transfusion or by other routes. The frequency of anti-Au(1) antibodies in sera from thalassaemic

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patients was much lower (2.1%) than in sera from haemophiliac patients (28.6%) (Blumberg, Alter, and Visnich, 1965), perhaps because the patients with haemophilia received fresh frozen plasma, whereas the patients with thalassaemia received packed red cells and whole blood.

We have found that there is an enormous variation in host response to infection, including the development of acute viral hepatitis, chronic and persistent active hepatitis, chronic anicteric hepatitis, and chronic infection (carrier state) with Au(1) without evidence of hepatitis (Blumberg *et al.*, 1970c). From this it is clear that host factors must be taken into account in evaluating the effects of transfusion.

In this paper we present results of a study of patients with thalassaemia living in northern Italy who have received large numbers of transfusions. This investigation has the epidemiological advantage that there is no problem of sampling; the entire sample group is included and the follow-up is nearly complete. We report our observations on the occurrence of Au(1) and anti-Au(1) antibody in 169 patients with thalassaemia. An average of three serum samples was tested annually during a period of 2 to 7 years. The largest number was 20. Age, sex, immunoglobulin levels, mortality rate, and splenectomy have been considered in their possible relation to the presence of Au(1) antigen and antibody.

Materials and Methods

A total of 3180 specimens from 169 thalassaemic patients collected over a 7-year period were studied. These patients included 143 from the Centro della Microcitemia di Ferrara; 21 from the Paediatric Clinic of the University of Ferrara; 3 from the Paediatric Clinic of the University of Siena; and 2 from the Paediatric Clinic of the University of Florence. This represents essentially the entire thalassaemia population attending these clinics during the period of observation.

Test for Au antigen and antibody.

(A) *Immunodiffusion.* The test was carried out in 8×10 cm plates, in 1.1% agarose in phosphate buffered saline (pH 7.4) (Blumberg *et al.*, 1970a). The method of Piazzi (1969) was also employed in the samples collected during 1969 and 1970 to determine the optimal conditions for the antigen/antibody reaction.

(B) *Cross-electrophoresis.* Australia antigen and antibody were also investigated in the sera collected during 1969 and 1970 by a modification of the reaction electrophoresis method of Lang (1966). 8×10 cm lantern slides were layered with 1% agarose (1.3% agar noble for the screening of antibodies) in barbitone buffer pH 8.2, 0.05 M. Two parallel rows of 2 mm diameter

holes were punched in the gel at a distance of 5 mm from each other. When the test is used for the detection of Au(1) antigen, the sera to be tested are placed in the wells punched in the cathodic portions of the slide. Antibody to Au(1) is placed in each opposite well. When detection of anti-Au(1) is desired, the unknown sera are placed in the anodic part of the slide, and a known positive Au(1) in the opposite wells. A current of 60 volts is then applied to the extremes of the slide, for a period of 75 minutes.

Quantitation of IgA, IgG, IgM, and IgD. The 18 thalassaemia patients with Au(1) in their serum were matched by sex in age order with 18 patients with anti-Au(1), and with 18 patients who had not developed either the antigen or the antibody. The age range for most triplets was three years, the maximum range for any triplet was six years. All sera were tested for immunoglobulin levels (IgA, IgG, IgM, IgD) by the method of Mancini, Carbonara, and Heremans, 1965, using commercial immunodiffusion plates (Melpar).

Statistical methods. The immunoglobulin values in the three groups of patients were compared by the Wilcoxon matched pair signed rank test. The relation between mortality, splenectomy, sex, and age, and the distribution of Au(1) antigen and antibody were tested by the χ^2 method, and Fisher's exact test. The sex distribution of Au(1) and anti-Au(1) was also evaluated using tests for the difference between two correlated proportions. When a total sample (n) is divided into several categories (x_1, x_2 , etc.), we can observe the proportions $x_1/n = p_1, x_2/n = p_2$, etc. To test the null hypothesis $p_1 = p_k = \bar{p}$, we expect $p_1 - p_k$ to be normally distributed with large enough n , and use the 2 statistics, with $2 = (\hat{p}_1 - \hat{p}_k) / \sqrt{2 \bar{p}/n}$. The Mann-Whitney test was used for evaluating mortality differences between the groups (Siegel, 1956).

Results

Frequency of Au(1) and anti-Au(1). Australia antigen was detected in 18 (10.6%) of the 169 thalassaemia children and the antibody in 35 (20.7%). In one case the antigen was observed before transfusion. The results with cross electrophoresis and the technique of Piazzi (1969) were the same as immunodiffusion, except that (1) antibody was detected in an additional 2 patients (35 instead of 33), and (2) in 9 patients, who had had Au(1) or anti-Au(1) detected by immunodiffusion in early samples; some follow-up samples were positive by cross electrophoresis though negative by immunodiffusion.

Persistence of antigen and antibody. Once present the antigen persisted for the duration of this study (2 to 7 years) or until the patient died

(Fig. 1). The only exception was the failure to detect Au(1) in patient PT in 1966 and 1968. Follow-up studies of the 35 patients with antibody are shown in Fig. 2. Antibody, in general, was persistent, but in several cases it disappeared, usually to return again. There was one case in which a weak antibody was found in a single sample,

FIG. 1.—Australia antigen in 18 patients with thalassaemia. Initials identify individual patients. Au(0) = absence of Australia antigen.

and antigen was subsequently detected in later samples. Other than this, the thalassaemic children had either antigen or antibody but not both.

Sex, age, mortality, and splenectomy. Au(1) is more frequent in males (14.3%) than in females (6.4%) (Table I). This difference is not statistically significant ($\chi^2 = 1.972$, $P > 0.10$), but it is consistent with findings in other healthy and disease populations (London, Sutnick, and Blumberg, 1969; Sutnick *et al.*, 1968; Blumberg *et al.*, 1970b; Blumberg *et al.*, 1972). Antibody is more frequent in females (30.0%) than in males (22.9%), but this difference is also not significant ($\chi^2 = 0.26$, $P > 0.50$). However, when the differences between two correlated proportions are tested we found that the proportion of females with anti-Au(1) was significantly greater than the proportion of females with Au(1) ($P = 0.00126$), whereas there was no significant difference between the proportion of males with Au(1) and those with anti-Au (1).

TABLE I
Distribution of Au(1) Antigen and Anti-Au(1) Antibody in Thalassaemic Children According to Sex

Sex	With Au(1)	With Anti-Au(1)	Without Au(1) or Anti-Au(1)	Total
Males	13	17	61	91
Females	5	18	55	78
Total	18	35	116	169

Tests for the difference between two correlated proportions:

(a) Proportion of males with Au(1) vs. proportion of males with anti-Au(1)

$z = 0.732$ $P = 0.76$, n.s.

(b) Proportion of females with Au(1) vs. proportion of females with anti-Au(1)

$z = -3.02$ $P = 0.00126$

(c) Proportion of both males and females with Au(1) vs. proportion of both with anti-Au(1)

$z = -2.37$ $P = 0.0176$

FIG. 2.—Antibody to Australia antigen in 35 patients with thalassaemia. Initials identify individual patients.

The average life span of patients with thalassaemia is 15 years. In order to determine the relation of age to the presence of Au(1) antigen and antibody, the study group was divided into those less than 7 years and those greater than 7 years (Table II).

TABLE II

Distribution of Au(1) Antigen and Anti-Au(1) Antibodies in Thalassaemic Children Divided in Two Groups According to Age

Age	With Au(1)	With Anti-Au(1)	Without Au(1) or Anti-Au(1)	Total
<7 yr	10	6	61	77
>7 yr	8	29	55	92
Total	18	35	116	169

Au(1) vs. anti-Au(1) with respect to age: $\chi^2 = 14.428$, $P < 0.001$.

TABLE III

Distribution of Au(1) and Anti-Au(1) in Thalassaemic Children by Age at First Blood Transfusion (Reliable data only)

Age at First Transfusion	With Au(1)	With Anti-Au(1)	Without Au(1) or Anti-Au(1) [Au(0)]	Total
> 12 mth	8	15	48	71
< 12 mth	9	14	47	70
Total	17	29	95	141

Median age (and ranges) at 1st transfusion (yr) 9/12 (3/12—7) 1 (3/12—5) 1 (3/12—20)

	P (Mann-Whitney)	P (χ^2)
Au(1) vs. anti-Au(1)	0.803	> 0.99
Au(1) vs. Au(0)	0.662	> 0.99
Anti-Au(1) vs. Au(0)	0.754	> 0.90

There is a higher frequency of antibody in the older patients and a higher frequency of antigen

in the younger ones, and this joint difference is significant ($\chi^2 = 17.410$, $P < 0.001$). At the time of the first blood transfusion, there were no differences between the ages of the patients who eventually developed Au(1), and anti-Au(1), or neither (Table III).

The mortality rate during the 7-year study period in patients with Au(1), and anti-Au(1) and with neither is shown in Table IV. It is higher in the patients with Au(1) (22.2%) than in those of the other groups (anti-Au(1) = 11.4%, neither Au(1) nor anti-Au(1) = 9.5%), and the median age at death is younger (Au(1) = 5 years, anti-Au(1) = 10.5 years, neither Au(1) nor anti-Au(1) = 7 years). However, the longer survival among the patients with anti-Au(1) is significant compared with the patients with Au(1) ($P = 0.014$), and is marginally significant compared with those without antigen or antibody ($P = 0.086$).

The relation of splenectomy to Au(1) and anti-Au(1) is shown in Table V. Treatment with

TABLE V

Frequency of Splenectomy in Thalassaemic Children in Relation to Presence or Absence of Au(1) Antigen or Anti-Au(1) Antibodies

Patients	No.	Splenectomy	%
With Au(1)	18	9	50
With anti-Au(1)	35	25	71
Without Au(1) or anti-Au(1)	116	53	46

Comparison of patients with Au(1) or anti-Au(1) vs. those with neither antigen nor antibody.

$\chi^2 = 7.149$, $p. < 0.05$.

splenectomy was most frequent in patients with anti-Au(1) and was more common in patients with Au(1) or anti-Au(1) than in the remaining patients with neither the antigen nor the antibody and the difference is significant ($\chi^2 = 7.149$, d.f. = 2,

TABLE IV

Mortality Rate in Thalassaemic Patients Observed Over a 5-year Period

Patients	No.	Dead	%	Median Age at Death (yr)	Age at Death Range (yr)
With Au(1)	18	4	22.2	5.0	(4-6)
With anti-Au(1)	35	4	11.4	10.5	(8-16)
Without Au(1) or anti-Au(1) [Au(0)]	116	11	9.5	7.0	(1-12)

P (Mann-Whitney)

Au(1) vs. anti-Au(1)	0.014
Au(1) vs. Au(0)	0.114
Anti-Au(1) vs. Au(0)	0.086

$P < 0.05$). However, splenectomy was performed in many instances after antibody was already present and, in general, on older children.

Immunoglobulins. Children with Au(1) or anti-Au(1) had significantly lower IgG immunoglobulin levels than those with neither antigen nor antibody. IgA levels were significantly lower in those with antigen than with antibody. No other significant differences were found, but levels of all immunoglobulins were slightly lower in those with Au(1) than with anti-Au(1) (Table VI).

Discussion

Patients with thalassaemia are subjected to repeated exposure to Australia antigen as a consequence of repeated blood transfusions. We have observed persistent antigen and antibody in them. We have not studied antigen/antibody complexes since the methods hitherto available for this measurement are either insensitive or indirect. The recent development by Coller *et al.* (1971) of a highly sensitive radioimmunoassay coprecipitation method which can apparently detect antigen/antibody complexes will now permit the biological study of significance of this reaction.

The frequency of Au(1) is relatively high in this thalassaemia patient group (10.6%), but not significantly higher ($\chi^2 = 2.445$, $P < 0.10$) than that reported for American thalassaemia patients (Blumberg *et al.*, 1965) from whom only single or small numbers of sera from each individual were tested. The antigen once present in these patients is nearly always persistent; there were only two inconsistencies in 61 patient years. The results are similar to those previously reported for apparently asymptomatic individuals living in Rongelap (Blumberg *et al.*, 1966), Down's syndrome patients (London *et al.*, 1969), lepromatous leprosy patients

living in Cebu (Blumberg and Melartin, 1970), and leukaemia patients (Sutnick *et al.*, 1970).

The persistence of Au(1) antigen in these patients contrasts with the situation in acute viral hepatitis in which the presence of the antigen is transient and may persist for only days or weeks (London *et al.*, 1969; Shulman and Barker, 1969). We have made the hypothesis that the persistence of the antigen is related to an immune impairment present in some patients (i.e. Down's syndrome, leukaemia) which may be associated with cellular immunity (Blumberg *et al.*, 1970c). It is not known if the persistence is due to recurrent infection of patients living in an environment where they are continually exposed to 'infection' with Australia antigen, or to the persistence of a single infection. Since these thalassaemic patients are transfused repeatedly they are continually re-exposed to the infection, and hence it is not possible to distinguish reinfection from persistent infection from these data.

The frequency of anti-Au(1) is very high in the patients reported here (20.7%). In thalassaemia patients transfused in Greece it is still higher (40.2%) (Edonomidou *et al.*, 1970), which might be due to a higher frequency of Au(1) among Greek blood donors than among Italian donors. Anti-Au(1) is sometimes persistent but less so than the antigen (Fig. 2). Individuals who develop persistent antibody are distinct from those who develop persistent antigen; that is, the ability to form antibody or antigen appears to be an inherent quality of the individual.

There are other characteristics that can define the differences between individuals who form persistent antibody, and those who develop persistent antigen. Males have a higher frequency of Australia antigen and females have a higher frequency of antibodies. The former observation is

TABLE VI

Comparison of Immunoglobulins in Respect of Australia Antigen and Antibody in Thalassaemia Patients (Wilcoxon Matched Pair Signed Ranks Test) (Mean Values Shown in mg/ml)

	IgG (mg/ml)	P	IgM (mg/ml)	P	IgA (mg/ml)	P	IgD (mg/ml)	P
Au(1)	13.7		1.27		1.54	<0.05	0.078	
Anti-Au(1)	16.9		1.62		2.19		0.158	
Au(1)	13.7	<0.01	1.27		1.54		0.078	
Au(0)*	20.0		1.58		2.40		0.0195	
Anti-Au(1)	16.9	<0.05	1.62		2.19		0.158	
Au(0)	20.0		1.58		2.40		0.0195	

*Au(0) = patients without Australia antigen or antibody.

consistent with our earlier observations that Au(1) is more common in males than in females in 22 of 23 populations studied (London *et al.*, 1969; Sutnick *et al.*, 1968; Blumberg *et al.*, 1970b; Blumberg *et al.*, 1972). The higher frequency of antibody in females has not been observed previously. Persistent antigen is more common among the younger patients, confirming our earlier observations in other populations (Blumberg, Sutnick, and London, 1968; Blumberg *et al.*, 1970c). Persistent antibody is commoner in older patients; this has not been studied previously.

The decreased immunoglobulin values may be related in part to the high frequency of splenectomy. We plan to test the hypothesis that transfused patients with thalassaemia may develop antigen/antibody complexes, and the decreased levels of immunoglobulin could be associated with this.

We had previously reported (Vierucci *et al.*, 1968) that the formation of anti-Ag (lipoprotein) antibodies contribute to the mortality in thalassaemic children. The increased mortality among the patients with Australia antigen is small and should be confirmed, but its validity is supported by the increased survival of the patients with anti-Au(1). If it is sustained, then we have identified a second factor which contributes to early mortality in thalassaemic children.

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