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Non-specific Binding of IgG to Antibody-coated Red Cells (The 'Matuhasi–Ogata Phenomenon')

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Summary. Several observers have reported that red cells coated with a specific blood-group antibody may take up a second blood-group antibody non-specifically, an effect known as the 'Matuhasi–Ogata phenomenon'. In the present work, this effect was investigated using either ^{125}I-labelled antibodies of various specificities or a ^{131}I-labelled preparation of IgG lacking relevant antibodies. In confirmation of much previous work, it was found that red cells took up appreciable amounts of IgG non-specifically; however, this uptake was not increased by previous coating of the red cells with specific antibody. When the IgG taken up non-specifically included a blood-group antibody in relatively high concentration, an eluate subsequently prepared from the red cells contained sufficient of the antibody to be detectable. Thus, the finding of unexpected antibodies in eluates may be due to non-specific uptake of IgG rather than to adherence of antibodies to antigen–antibody complexes.

INTRODUCTION

Matuhasi (1959) suggested that antibodies of one specificity might adhere to antigen–antibody complexes of a different specificity and further evidence was published by Ogata and Matuhasi (1962, 1964). Since then this effect, now known as the Matuhasi–Ogata phenomenon, has been reported with sera containing warm- and cold-acting autoantibodies (Svardal, Yarbro and Yunis, 1967; Allen, Issitt, Degnan, Jackson, Riehart, Knowlin and Adebahr, 1969), and alloantibodies of many specificities including immune and naturally-occurring anti-A or anti-B (Allen *et al.*, 1969), and with the precipitate formed between syphilitic antibody and VDRL antigen (Ogata and Matuhasi, 1964). The phenomenon has been seen with whole serum and with its IgM and IgG fractions and has been considered responsible for, among other things, loss of desired antibody during the absorption of serum to prepare blood-typing reagents.

The experiments described in this paper were designed to study the Matuhasi–Ogata phenomenon using labelled antibodies of different specificities and labelled IgG lacking demonstrable blood-group specificity.

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MATERIALS AND METHODS

Antisera

Most antisera, some of which had been stored frozen, were from patients who had been immunized by transfusion or pregnancy. The anti-Le^a came from a group A donor immunized by injection of purified human Lewis substance; the serum agglutinated Le(a+) red cells both at room temperature and at 37° and, at a dilution of 1 in 512, sensitized red cells to agglutination by anti-complement-globulin serum; some anti-Le^b activity was present in the serum. Anti-I (Rob) came from a hospitalized patient who had neither haemolytic anaemia nor cold agglutinin disease; the serum agglutinated adult red cells strongly at room temperature and in the cold, but had a titre of only 4. Anti-I (Steph) was from a patient with a 20-year history of cold agglutinin disease; the titre was 50,000 at 4° and 25,000 at room temperature; in the cold, the serum haemolysed adult red cells to a slight degree.

The labelled anti-D was prepared from pooled serum from hyperimmunized volunteers. The labelled anti-A was prepared from the plasma of a group O person stimulated by the injection of purified human A substance. In each case, the IgG fraction was isolated by elution from DEAE cellulose using 0·02 M Tris buffer, pH 8·0, concentrated by ultrafiltration, and labelled with ^{125}I by the method of McFarlane (1958). The labelled anti-A was used without further processing and had an IgG concentration of 7·18 mg/ml and an initial specific activity of $13{\cdot}0 \times 10^6$ cpm/mg.

The labelled anti-D was adsorbed on to D-positive red cells and eluted by the method of Rubin (1963). The red cells were then washed and a second eluate prepared by the same method. In the second eluate, 48 per cent of the protein was anti-D; and the uptake of radioactivity on to D-negative red cells was $<0{\cdot}3$ per cent of the uptake on to D-positive cells.

'Non-antibody' IgG

Plasma lacking demonstrable blood-group antibodies was collected from four group AB blood donors. The IgG fraction was isolated from this pooled plasma by elution from DEAE cellulose, using 0·02 M Tris buffer, pH 8·0, concentration by ultrafiltration, and labelled with ^{131}I by the method of McFarlane (1958). In the final preparation, the concentration of IgG was 5·0 mg/ml and the initial specific activity $3{\cdot}0 \times 10^6$ cpm/mg.

Red cells

Blood collected from normal volunteers was stored in ACD solution and used within 7 days of collection. Red cells were washed four times in 0·17 M saline; 50 μl of red cells were used for each incubation mixture in experiments with anti-A IgG or 'non-antibody' IgG and 100 μl for the experiments with anti-D.

Reaction conditions

In experiments with labelled anti-A IgG or 'non-antibody' IgG, three dilutions were prepared in 3 per cent bovine serum albumin in buffered saline. When both anti-A IgG and 'non-antibody' IgG were added to the packed cells, equimolar amounts of each were used. All reaction mixtures were brought to a volume of 1·0 ml with 3 per cent bovine serum albumin in saline before incubation.

In most experiments, red cells were first incubated with non-labelled antibody at 37° for 1 hour. The anti-I was used at 4° or at 20° and the anti-Le^a at 20°. Incubation with unlabelled AB serum in the place of antibody was carried out as a control for all experiments. After incubation, the cells were washed four times with ice-cold saline before adding the labelled antibody or 'non-antibody' IgG. In all cases the antiglobulin test was positive or the cells were strongly agglutinated before the addition of labelled antibody or 'non-antibody' IgG.

In experiments with labelled anti-A, group O red cells were tested in parallel with the group A cells. The uptake of labelled anti-A on the O cells was considered non-specific and subtracted from the amount taken up by A cells to give values for uptake of anti-A. In experiments with labelled 'non-antibody' IgG, all radioactivity bound to red cells is non-specific in an immunological sense. The non-specific material includes some IgG, lipid and aggregated immunoglobulin (Hughes-Jones and Gardner, 1962).

For absorption and elution experiments, 1·0 ml of well-washed, packed red cells was incubated with 2·0–2·5 ml of serum at 37° for 1–2 hours. The cells were washed four times with ice-cold saline, and an equal volume of 3 per cent bovine serum albumin in saline added. Elution was at 56° for 10 minutes with constant agitation, followed by centrifugation in a heated centrifuge. The eluate was removed promptly and tested the same day by the antiglobulin method. Six to 10 volumes of eluate were added to one volume of a 2 per cent suspension of well-washed cells. After 1 hour at 37°, the cells were washed three times with ice-cold saline and two drops of broad spectrum antiglobulin serum added to the cell button. After mixing, the tubes were centrifuged lightly and read macroscopically.

As suggested by Allen *et al.* (1969), we have used the term 'right' to indicate antibody that fixes to red cells possessing the appropriate blood-group specificity and 'wrong' for antibody reacting with red cells lacking the appropriate specificity.

RESULTS

The specific uptake of labelled anti-A by group A, D-positive red cells previously sensitized with anti-D was essentially the same as that of the same cells previously incubated with normal serum (Fig. 1). Results were similar with the six different concentrations of anti-A tested.

When group O, D-positive red cells were incubated with labelled anti-A IgG, there was, as previously reported (Boursnell, Coombs and Rizk, 1953), non-specific uptake of labelled protein (see Fig. 2). As was the case with specific uptake, however, previous sensitization of the cells with anti-D did not enhance subsequent uptake of radioactivity from the labelled anti-A. Rather, there was a suggestion that previous sensitization with anti-D diminished the subsequent uptake of labelled protein, particularly at higher concentrations.

Thus, neither the specific uptake of antibody nor the non-specific uptake of labelled IgG was enhanced by previous sensitization of the red cells.

To study further the possibility that previously sensitized cells might show increased binding of IgG, either with or without blood-group specificity, sensitized and control cells were incubated with mixtures containing equal amounts of ^{125}I-anti-A and ^{131}I-'non-antibody' IgG. Results with total ($^{131}I + ^{125}I$) IgG concentrations of 7·2, 2·8 and 1·4 mg/ml were similar and showed that previous sensitization of group A, D-positive red cells with anti-D did not increase the subsequent uptake of anti-A or of IgG (see Table 1).

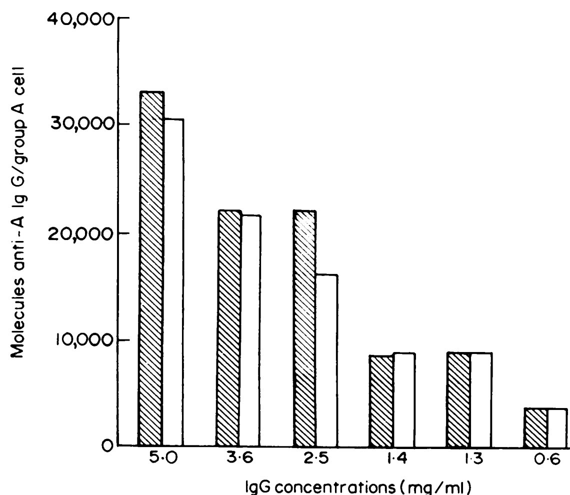

FIG. 1. Effect of previous sensitization with anti-D on the number of molecules of anti-A IgG bound per group A, D-positive red cell after incubation with six different concentrations of anti-A IgG. The values represent uptake of anti-A IgG after correction for non-specific uptake of IgG by group O red cells. Diagonal hatched bars, cells sensitized with anti-D. Open bars, control cells.

In these experiments, in which equal amounts of labelled 'non-antibody' IgG and of labelled anti-A IgG were present, the total non-specific uptake on group A cells was about twice that seen when red cells were incubated with anti-A alone.

D-positive red cells took up approximately 3660 molecules of anti-D per cell when incubated with the highly purified preparation of antibody (see Table 2). In comparison, D-negative cells took up fewer than 10 molecules per cell under the same conditions. In all the cases but one, previous sensitization of these cells with anti-Le^a, -Fy^a, -c, -e, -B, -AB or -I did not increase the uptake of labelled anti-D. Results were not influenced by the fact that the anti-D itself was the 'right' antibody in two cases and the 'wrong' antibody in seven (see Table 2).

In one case, group O, D-negative cells sensitized with a potent anti-I (Steph) were

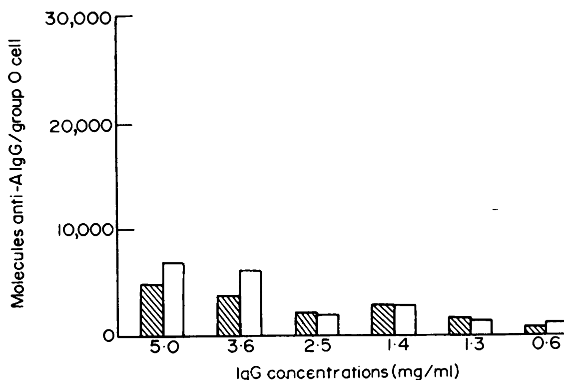

FIG. 2. Effect of previous sensitization with anti-D on the number of molecules of IgG bound per group O, D-positive red cell after incubation with six different concentrations of anti-A IgG. The values represent non-specific uptake from a preparation of anti-A IgG. Diagonal hatched bars, cells sensitized with anti-D. Open bars, control cells.

TABLE 1

EFFECT OF PREVIOUS SENSITIZATION WITH ANTI-D ON THE NUMBER OF MOLECULES OF IgG SUBSEQUENTLY BOUND PER GROUP A, D-POSITIVE RED CELL AFTER INCUBATION WITH MIXTURES OF EQUAL AMOUNTS OF 'NON-ANTIBODY' IgG AND ANTI-A IgG

	Molecules of IgG bound					
	7·2 mg IgG/ml*		2·8 mg IgG/ml*		1·4 mg IgG/ml*	
	Sensitized†	Control†	Sensitized†	Control†	Sensitized†	Control†
Anti-A IgG	21187	20241	9606	9937	3119	4339
Non-specific IgG	10317	12128	3737	2969	2974	2579
Total IgG	31504	32369	13343	12906	6093	6918

* Fifty per cent anti-A IgG and 50 per cent 'non-antibody' IgG.
† Sensitized cells previously incubated with anti-D; control cells incubated with AB serum.

strongly agglutinated and partially lysed before the labelled anti-D was added. These cells took up slightly more antibody than control cells, but only 2 per cent of the amount taken up by D-positive cells (see Table 2).

The uptake of anti-A on group A, Le(a+) red cells was no greater when the cells were previously sensitized with anti-Le^a than when they were incubated with AB serum (see Table 3). The addition of equimolar amounts of 'non-antibody' IgG to the antibody IgG increased the total amount of IgG bound but, again, control and Le^a-sensitized cells took up similar amounts.

The number of molecules of labelled IgG taken up by group O cells previously sensitized with anti-I and then incubated with either labelled anti-A IgG or labelled 'non-antibody'

TABLE 2

MOLECULES OF ANTI-D BOUND TO D-NEGATIVE AND D-POSITIVE RED CELLS PREVIOUSLY SENSITIZED WITH BLOOD-GROUP ANTIBODIES OF VARIOUS SPECIFICITIES

	Unsensitized	Sensitized with anti-						
		Fy^a	c	e	Le^a	AB	B	I (Steph)
D-negative	7	8	7	6	3	19	11	62
D-positive	3660	—	—	—	3700	—	—	3560

TABLE 3

EFFECT OF PREVIOUS SENSITIZATION WITH ANTI-Le^a ON THE NUMBER OF MOLECULES OF IgG SUBSEQUENTLY BOUND PER GROUP A, Le(a+) RED CELL AFTER INCUBATION WITH DIFFERENT AMOUNTS OF ^{125}I-LABELLED ANTI-A IgG

	Molecules of IgG bound					
	3·6 mg IgG/ml		1·4 mg IgG/ml		0·7 mg IgG/ml	
	Sensitized*	Control*	Sensitized*	Control*	Sensitized*	Control*
Anti-A IgG	18663	21539	7312	8681	3473	3764
'Non-antibody' IgG	2284	2488	1275	1265	710	861
Total IgG	20947	24027	8587	9946	4183	4625

* Sensitized cells previously incubated with anti-Le^a; control cells incubated with AB serum.

TABLE 4

EFFECT OF PREVIOUS SENSITIZATION WITH ANTI-I ON THE NUMBER OF MOLECULES OF IgG SUBSEQUENTLY BOUND PER GROUP O, I-POSITIVE RED CELL AFTER INCUBATION WITH ANTI-A IgG AND 'NON-ANTIBODY' IgG

	Previously sensitized with anti-I	Control
Anti-A IgG (3·6 mg/ml)	5594	5187
'Non-antibody' IgG (3·6 mg/ml)	9210	7232

TABLE 5

ELUTION OF THE 'WRONG' ANTIBODY AFTER INCUBATION OF HIGH TITRE SERA WITH RED CELLS LACKING THE APPROPRIATE ANTIGEN

Serum			
Specificity (donor)	Indirect antiglobulin titre	Red cells*	Antibody in eluate
Anti-D (Avg)	2000	O, D-negative	Anti-D
Anti-D (Au)	2000	O, D-negative	Anti-D
Anti-B	4000	O, D-positive	Anti-B

* Incubated with serum and then used for preparation of eluate.

IgG is shown in Table 4. The Table gives results for one experiment; similar results were seen with two examples of anti-I, each used at three concentrations. There was no preferential uptake of labelled anti-A; in fact, at all concentrations tested, the uptake of 'non-antibody' IgG was greater than that of anti-A IgG although the differences were relatively small and probably within the error of the method. Uptake onto I-sensitized red cells was not significantly greater than uptake onto uncoated red cells.

The foregoing experiments indicated that the explanation previously offered for the Matuhasi–Ogata phenomenon might be incorrect. Thus, it had been supposed that uptake of the 'wrong' antibody by red cells previously coated with a 'right' antibody was due to some interaction between the two antibodies, whereas the experiments reported here indicated that previous sensitization of red cells with antibody did not affect the subsequent uptake of IgG molecules. The alternative possibility was therefore considered, that the presence of the 'wrong' antibody in eluates might be due simply to the non-specific uptake of IgG on to red cells. When the non-specific IgG included a 'wrong' antibody, this antibody might subsequently be detected in an eluate prepared from the cells. In order to test this hypothesis, well-washed red cells were incubated with serum containing only a 'wrong' antibody. Subsequent testing of the eluates, using appropriate controls, showed the presence of the 'wrong' antibody (see Table 5).

DISCUSSION

The experiments described here were designed to study the adhesion of antibodies of one specificity to an antigen–antibody complex containing antibody of a different speci-

ficity. Previous workers have apparently obtained evidence of this phenomenon either by finding a fall in titre of a 'wrong' antibody coincident with the adsorption of a 'right' antibody or by the elution of a 'wrong' antibody from a red cell–antibody complex. To date, however, there has been no direct evidence that the unexpected antibody is, in fact, present on the antigen–antibody complex.

Our data show that, compared with unsensitized red cells, cells sensitized with various warm-acting antibodies do not take up increased amounts of IgG. Because of a suggestion that dilution of the serum might increase the likelihood of non-specific adsorption (Allen *et al.*, 1969), anti-A concentrations from 0·6–5·0 μg/ml of serum were studied. The lower dilutions, used at a serum: cells ratio of 20: 1, represent amounts of IgG antibody adequate to cause a positive antiglobulin test, but are below the amount usually required to bring about weak agglutination. At these levels, as well as at levels sufficient to react with about 1 per cent of the available A sites, control and sensitized red cells took up identical amounts of labelled anti-A. The results with labelled anti-D, after the cells had been sensitized with antibodies such as anti-c, anti-e, etc. in relatively high concentrations, were similar and gave no indication that previous sensitization increased the uptake of labelled antibody.

The suggestion that the phenomenon of non-specific adhesion might be temperature-dependent has not been confirmed. Our experiments with group O, I-positive red cells coated with anti-I (a 'cold' antibody) showed no evidence of greater uptake of labelled anti-A compared with control red cells. In the one case in which I-sensitized red cells did show enhancement of uptake, there was both strong agglutination and slight lysis, and even here uptake was only 2 per cent of that seen with unsensitized D-positive red cells. This slight increase seems best explained by the trapping of labelled antibody in the large agglutinates.

The possibility that the Matuhasi–Ogata phenomenon might be explained by the normal coating of protein, especially IgG, on red cell surfaces rather than adhesion of antibody to a pre-formed antigen–antibody complex was investigated with ^{131}I-labelled 'non-antibody' IgG used in conjunction with ^{125}I-labelled anti-A IgG. In such experiments, non-specific binding of IgG should be evidenced by equal uptakes of labelled antibody and IgG; moreover, it would be expected that uptake of IgG would not be increased by previous sensitization of red cells with antibody. If, on the other hand, antibody molecules are preferentially bound to immune complexes, the amount of anti-A IgG taken up by sensitized cells should be greater than the amount of 'non-antibody' IgG. Our data show no difference in total uptake when equimolar amounts of labelled anti-A IgG and 'non-antibody' IgG were incubated with sensitized or control cells. As expected, more anti-A IgG than 'non-antibody' IgG was fixed by A cells, but in all cases previous sensitization of the red cells (e.g. by anti-D) neither increased the total uptake of IgG nor the percentage of bound IgG that was blood-group specific. Group O cells, incubated with anti-A IgG, 'non-antibody' IgG, or both, took up essentially the same amount of IgG after sensitization with anti-I as did control cells. At higher concentrations there was even a suggestion that anti-I inhibited, rather than enhanced, subsequent uptake of anti-A (when compared to 'non-antibody' IgG), but the differences may be within the range of error of the method. If further experiments confirm this finding, it will be additional evidence of the close relationship of the A and I antigens reported by Feizi, Kabat, Vicari, Anderson and Marsh (1971).

There is considerable evidence that IgG is present on normal red cells. Gergely, Arky and Medgyesi (1967) have reported that this non-specifically-bound IgG consists only of

those IgG molecules that are papain-insensitive, while Fidalgo, Najjar and Overall (1967) have shown that IgG fractions with varying affinity for normal red cells can be separated by chromatography on cellulose phosphate. In an experiment conducted at low ionic strength, Harshman and Najjar (1963) were able to elute anti-A or anti-B but not anti-viral antibodies from red cells that had been exposed to normal serum containing all three antibodies. In our experiments, red cells incubated with IgG took up between 575 and 17,500 molecules of IgG per cell in a non-specific fashion, and there was no suggestion that IgG with blood-group specificity was selectively bound. Uptake was greater at 4° than at 37° and, at all temperatures, was related directly to the concentration of IgG in the incubation mixture.

It is entirely possible that the Matuhasi–Ogata phenomenon can be explained, at least in some cases, on the basis of the non-specific uptake of IgG by red cells. When the IgG taken up by the red cells includes molecules with blood-group specificity, an eluate prepared subsequently from the cells will contain these molecules together with other IgG molecules which have combined non-specifically with the cells. If these non-specifically-bound antibody molecules are present in sufficient concentration, they may be detectable in the eluate. As discussed by Voak and Williams (1971) and as recently emphasized by Romano, Hughes-Jones and Mollison (1973), the elution procedure as commonly performed does, in fact, result in a considerable concentration of antibody molecules. Thus, even when relatively small amounts of antibody are present on the red cells from which the eluates are made, if the whole eluate is subsequently incubated with a very small volume of red cells (as is usually the case), the number of antibody molecules per cell may be fifty times higher on the final test cells than on the cells from which the eluate was made (Romano *et al.*, 1973).

One can consider a hypothetical case in which a serum with an IgG concentration of 1·2 g/100 ml contains anti-D with a concentration of 40 μg/ml, so that 0·33 per cent of the IgG molecules have anti-D specificity. Now, suppose that 1 ml of D-negative red cells is incubated with this serum and an eluate is subsequently made. Normal red cells can bind non-specifically about 10 μg IgG/ml red cells and, of this, assume that 60 per cent can be recovered in an eluate; an eluate with a volume of 1 ml would then contain about 6 μg IgG. Since 0·33 per cent of this IgG is anti-D, there could be 0·02 μg anti-D in the eluate, which is an amount which is readily detectable in the antiglobulin test (Mollison, 1972). As Table 5 in the present paper shows, it is in fact possible to detect anti-D in eluates made in just this way; similarly, anti-B can be detected in eluates made from group O cells previously incubated with anti-B.

In conclusion, both on theoretical grounds and as demonstrated by experiment, non-specific adsorption of immunoglobulin to red cells seems to provide a satisfactory explanation for the 'Matuhasi–Ogata phenomenon'.

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REFERENCES

Allen, F. H., jr, Issitt, P. D., Degnan, T. J., Jackson, Valerie A., Reihart, Judith K., Knowlin, R. J. and Adebahr, Margot E. (1969). 'Further observations on the Matuhasi–Ogata phenomenon.' *Vox Sang. (Basel)*, **16**, 47.

Boursnell, J. C., Coombs, R. R. A. and Rizk

VIVIEN (1953). 'Studies with marked antisera. Quantitative studies with antisera marked with iodine ^{131}isotope and their corresponding red-cell antigens.' *Biochem. J.*, **55**, 745.

FEIZI, TEN, KABAT, E. A., VICARI, G., ANDERSON, B. and MARSH, W. L. (1971). 'Immunochemical studies on blood groups. XLVII. The I antigen complex—precursors in the A, B, H, Le^a, and Le^b blood group system—hemagglutination-inhibition studies.' *J. exp. Med.*, **133**, 39.

FIDALGO, B. V., NAJJAR, V. A. and OVERALL, J. (1967). 'Abnormal chromatographic pattern of γ-globulin in acute rheumatoid arthritis.' *Johns Hopk. med. J.*, **121**, 73.

GERGELY, J., ÁRKY, I. and MEDGYESI, G. A. (1967). 'Nonspecific interaction of the papain-insensitive and papain-sensitive populations of human IgG with erythrocytes.' *Vox Sang. (Basel)*, **12**, 252.

HARSHMAN, S. and NAJJAR, V. A. (1963). 'The binding of autologous γ-globulin with isohemagglutinin activity to human red blood cells.' *Biochem. biophys. Res. Commun.*, **11**, 411.

HUGHES-JONES, N. C. and GARDNER, BRIGITTE (1962). 'The exchange of ^{131}I-labelled lipid and ^{131}I-labelled protein between red cells and serum.' *Biochem. J.*, **83**, 404.

MCFARLANE, A. S. (1958). 'Efficient trace-labelling of proteins with iodine.' *Nature (Lond.)*, **182**, 53.

MATUHASI, T. (1959). 'Plasma protein and antibody fractions observed from the serological point of view.' *Proc. 15th Gen. Assembly Jap. med. Congr., Tokyo*, 1959, **4**, 80.

MOLLISON, P. L. (1972). *Blood Transfusion in Clinical Medicine*, p. 283, 5th edn. Blackwell Scientific Publications, Oxford.

OGATA, T. and MATUHASI, T. (1962). 'Problems of specific and cross reactivity of blood group antibodies.' *Proc. 8th Congr. int. Soc. Blood Transf., Tokyo* 1960, 208.

OGATA, T. and MATUHASI, T. (1964). 'Further observations on the problem of specific and cross reactivity of blood group antibodies.' *Proc. 9th Congr. int. Soc. Blood Transf., Mexico* 1962, 528.

ROMANO, E. L., HUGHES-JONES, N. C. and MOLLISON, P. L. (1973). 'Direct antiglobulin reaction in ABO-haemolytic disease of the newborn.' *Brit. med. J.*, **i**, 524.

RUBIN, H. (1963). 'Antibody elution from red blood cells.' *J. clin. Path.*, **16**, 70.

SVARDAL, JANET M., YARBRO, J. and YUNIS, E. J. (1967). 'Ogata phenomenon explaining the unusual specificity in eluates from Coombs-positive cells sensitized by autogenous anti-I.' *Vox Sang. (Basel)*, **13**, 472.

VOAK, D. and WILLIAMS, M. A. (1971). 'An explanation of the failure of the direct antiglobulin test to detect erythrocyte sensitization in ABO haemolytic disease of the newborn and observations on pinocytosis of IgG anti-A antibodies by infant (cord) red cells.' *Brit. J. Haemat.*, **20**, 9.