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BLOOD GROUP ANTIBODIES AS A SOURCE OF ERROR IN THE DIAGNOSIS OF PEMPHIGUS BY INDIRECT IMMUNOFLUORESCENCE

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Abstract. Sera from 580 patients suffering from miscellaneous skin diseases were tested by indirect immunofluorescence (IFL) for antibodies reacting with epithelial intercellular substance, using monkey oesophagus mucosa as the substrate. Sera from 43 patients gave a positive reaction. After absorption with A and B erythrocytes, sera from only five cases remained positive. The clinical diagnosis in these 5 patients was: pemphigus vulgaris in 2 patients, pemphigus erythematosus in 2 and probable pemphigus in 1 case. Thus 38 (7%) of the examined sera showed a "false positive" IFL reaction for pemphigus due to the presence of antibodies to blood group substances. Blood group antibodies demonstrated by IFL were of IgG class and in some sera also of IgA class whereas hemagglutinating anti-A and B antibodies belonged to the IgM class. Ten out of 26 sera selected because of elevated titres of antistreptolysin also showed a false positive IFL reaction to pemphigus. For the diagnosis of pemphigus by IFL all sera reacting with epithelial intercellular substance should be reexamined after absorption with A and B erythrocytes.

It has been shown that sera from patients suffering from pemphigus contain antibodies against the epithelial intercellular substance of skin and mucous membranes, demonstrable by the indirect immunofluorescent (IFL) technique (2, 3). A similar pattern of fluorescence has also been demonstrated with sera from patients with severe burns (2).

Blood group antigens are present as surface components in many epithelial and vascular endothelial cells (6, 9). Grob & Inderbitzin (5, 7) found that "immune sera" against A and B blood group substances, when tested by the IFL-technique on tissue sections of rabbit oesophagus, gave a reaction similar to pemphigus sera. The reactivity was lost after absorption with group A and group B erythrocytes, whereas pemphigus sera retained their reactivity after absorption. This "false positive" reaction was not observed with so-called normal human sera containing anti-A or anti-B antibodies, and it was thus considered to be of no importance as an error in the diagnosis of pemphigus by indirect IFL.

In routine diagnostic testing of sera for pemphigus antibodies we observed that sera from several patients with miscellaneous skin diseases but without clinical signs of pemphigus gave a positive IFL reaction with epithelial intercellular substance. The present study was undertaken to determine if this reaction was due to blood group antibodies.

MATERIALS AND METHODS

Sera

Sera from 580 patients with miscellaneous skin diseases, sent to the laboratory during the period January 1969 to April 1971 were investigated by IFL in a dilution of 1/10. Positive sera were titrated twofold from 1/20 to 1/320 and examined by IFL. The positive sera were also absorbed with group A and group B erythrocytes and thereafter again tested by indirect IFL.

26 sera with an elevated antistreptolysin titre (AST 800–1 600) (8) were also examined by IFL.

Tissue sections

The oesophagus of Macaca fascicularis monkeys of blood group A or AB was used as the substrate for IFL testing. Small tissue blocks were quick frozen in solid CO_2 and kept at – 70°C until used. Sections, 6–8 μm in thickness, were cut in a cryostat. The sections were used unfixed.

Conjugates

A fluoresceinisothiocyanate (FITC)-conjugated sheep IgG against human immunoglobulins prepared at the Department of Immunology, National Bacteriological Laboratory (SBL) was used. This conjugate contained 7.0 mg protein/ml and 3.0 mg anti IgG/ml. The molar fluorescein/protein (F/P) ratio was 2.5. It was standardized as described by Beutner (2). It was used in dilution 1 : 15.

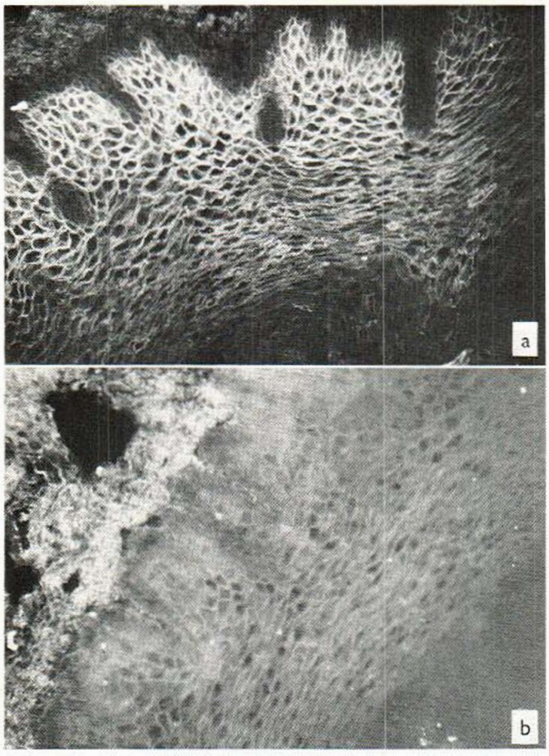

Fig. 1. (*a*) False positive IFL reaction to pemphigus. Epithelial intercellular substance in monkey oesophagus mucosa stained in the first step with serum from a patient with a skin disease and in the second step with a FITC-conjugated anti human gammaglobulin. (*b*) Negative reaction after absorption of the patient's serum with A and B erythrocytes.

The immunoglobulin classes of antibodies in some sera were determined by use of FITC-conjugated monospecific immunoglobulins to IgM, IgA and IgG respectively. The monospecific sheep anti-IgM and IgA conjugates were prepared at the Department of Immunology, SBL. The anti-IgM conjugate contained 7.0 mg protein/ml, antibody protein 0.55 mg/ml and its F/P ratio was 2.9. It was used in dilution 1 : 8. The IgA conjugate contained 9.5 mg protein/ml antibody protein 0.9 mg/ml. Its F/P ratio was 3.1. It was used in a dilution of 1 : 5. The FITC conjugated sheep antihuman IgG was purchased from Wellcome Laboratories (Beckenham, England) and contained 9.8 mg protein/ml, 0.4 mg antibody protein/ml and its F/P ratio was 3.2. It was used in a dilution of 1 : 8.

IFL-test

The indirect IFL-test was performed essentially as described by Coons & Kaplan (4). The tissue sections were treated with diluted serum, rinsed in phosphate-buffered saline, treated with conjugate, rinsed again and then mounted in buffered glycerine. The preparations were examined in a Zeiss fluorescence microscope equipped with a HBO 200 mercury lamp and a darkfield condenser. Primary filters BG 3 and secondary filters 44 or 44 and 47 were used.

Hemagglutination (HA)

For the determination of antibody titres against blood groups A and B, sera were diluted twofold in 0.025 ml volumes with the microtitre technique in disposable plastic dishes. An equal volume of a 1% suspension of A or B erythrocytes was added and the plates were read after incubation for 1 hour at room temperature.

Absorption of blood group antibodies

Equal volumes of undiluted serum and packed A or B erythrocytes were mixed and kept at room temperature for 30 min with occasional shaking. The mixture was then centrifuged and the supernatant examined by IFL and HA. After absorption with A or B erythrocytes, the HA titre against the corresponding erythrocytes was < 2.

Density gradient ultra-centrifugation

Three sera were fractionated by density gradient ultracentrifugation. A gradient of sucrose was prepared ranging from 10–37%. ●.4 ml of heated serum (56°C, 30 min) diluted 1 : 4 in PBS was layered over the gradient to obtain a final volume of 5 ml, which was then centrifuged in a Spinco centrifuge using a SW 50 rotor at 35 000 rpm for 18 hours at 5°C. Eleven serial fractions of approximately 0.45 ml were collected dropwise from the bottom of the tubes and examined for antibody acivity. The separation of IgM, IgA and IgG was checked by testing the fractions by immunodiffusion according to Ouchterlony, against a monospecific antiserum to IgG, IgM and IgA respectively.

Mercaptoethanol treatment

Sera or serum fractions were incubated for 1 hr at 37°C with an equal volume of 0.2 M mercaptoethanol in phosphate-buffered saline and with the buffer alone and thereafter tested for antibody activity.

RESULTS

Sera from 58● patients with various skin diseases were examined by IFL for antibodies against epithelial intercellular substance. Forty-three of these sera were positive. After absorption with A- and B-erythrocytes only five sera remained positive. Four of these sera, containing antibodies to intercellular substance in titres ranging from 10 to 160, were taken from patients showing clinically and histologically typical pemphigus vulgaris (two cases) and pemphigus erythematosus (two cases). The fifth patient was diagnosed as a case of lichen ruber planus of the mouth. She may have been suffering from pemphigus of the mucous membranes. Her serum contained antibodies to intercellular substance in a titre of 320.

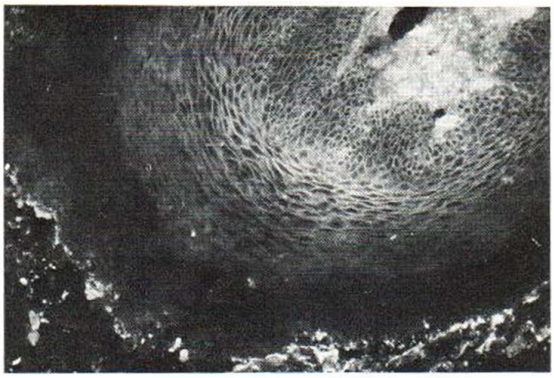

Fig. 2. Positive IFL reaction to pemphigus. Staining as in Fig. 1 *a* but with serum from a patient suffering from pemphigus.

Thus 38 (7%) out of 580 examined sera showed a false positive IFL reaction to pemphigus. Fig. 1 shows the results of IFL staining of monkey oesophagus epithelium with one serum before and after absorption with A and B erythrocytes. For comparision, Fig. 2 shows the IFL reaction obtainined with a serum from a patient with pemphigus.

The IFL titres of the sera reacting with intercellular blood group substance ranged from 10 to 160 as shown in Fig. 3. The HA antibody titres of these sera against A and B erythrocytes ranged from < 2 to 2 048 (Fig. 3). Figure 3 demonstrates an absence of correlation between the IFL antibody titres against intercellular blood group A substance and the HA titre against A erythrocytes.

The possihility was considered that this lack of correlation was due to differences in the immunoglobulin classes of the anti-A antibodies as measured by the two serological methods. Eight sera were, therefore, examined by IFL with the use of monospecific conjugates against IgM, IgG and IgA, respectively. Two of these sera had IFL titres of 80 and 160, respectively, but HA titres of only 4. All of these eight sera were found to have IFL antibodies of IgG class to blood group A intercellular substance but no demonstrable IFL antibodies of IgM class. Two of the sera also contained antibodies of IgA class.

Three of the sera which had been shown to have IFL antibodies of IgG class and which had high anti-A HA titres were fractionated into 19 S (IgM) and 7 S (IgG and IgA) immunoglobulins by density graident centrifugation. The results obtained with one of these sera are shown in Fig.

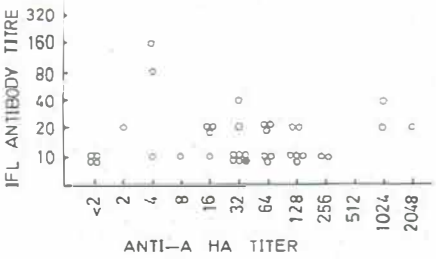

Fig. 3. Titres of IFL antibodies reacting with epithelial intercellular substance of a blood group A monkey oesophagus and of anti-A HA titres in 38 sera from patients with miscellaneous skin diseases.

4. The fractions were tested by immunodiffusion against monospecific antisera to IgG, IgM and IgA. Fractions 1 and 2 gave a precipitin line with IgM antisera, fractions 4–8 reacted with anti-IgG and fractions 3–7 with anti-IgA. In all three fractionated sera the main HA antibody activity was recovered in the fractions containing IgM (Fig. 4; fractions 1–2). There was some activity in the third fraction but no HA antihodies in the IgG fractions. Treatment of the fractions with 2-mercaptoethanol abolished the HA antibody activity, indicating that the antibodies were IgM.

Ten out of 26 sera with a high AST titre ($\geqslant 800$) also gave a positive IFL reaction with epithelial intercellular substance, which could be abolished by absorption with A and B erythrocytes. The anti-A and anti-B agglutination titres of these sera were fairly low (< 2–128).

COMMENTS

In this investigation we found that 7% of sera from patients suffering from various skin diseases

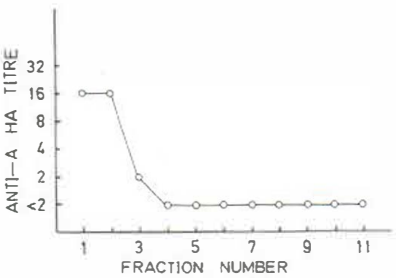

Fig. 4. Distribution of HA antibodies against A erythrocytes in a serum fractionated by density gradient centrifugation. The fractions were collected from the bottom of the centrifuge tube.

other than pemphigus stained the intercellular substance of monkey oesophagus tissue when investigated by the indirect IFL technique. This staining disappeared after absorption of the sera with A and B erythrocytes. Similar results have recently been reported by Andersson et al. (1). These authors demonstrated intercellular fluorescence with sera from 24 out of 75 patients with diseases other than pemphigus. The staining was abolished by absorption of sera with blood group B substance. Both in the study by Andersson and co-workers and in our study there was a poor correlation between the titres of blood group isohemagglutinins and of IFL antibodies. We were able to show that the antibodies demonstrated by IFL were of IgG class and to some extent of IgA class, whereas the anti-A and anti-B HA antibodies belonged to the IgM class. This finding can explain the lack of correlation between the IFL and HA antibody titres against blood group substance.

Sera with a high titre of AST also gave a false positive IFL reaction to pemphigus in a high percentage (39%). It is possible that some streptococcal antibodies cross-react with blood group antigens.

The present study has shown that blood group antibodies are a source of error in the diagnosis of pemphigus by IFL. It is recommended that all sera giving a positive reaction with epithelial intercellular substance should be retested after absorption with A and B erythrocytes.

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