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THE INFLUENCE OF DIFFERENT TECHNIQUES IN CHARACTERIZING HUMAN ANTIBODIES TO COW'S MILK PROTEINS

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SUMMARY

Sera from 760 subjects with and without inflammatory bowel disease (IBD) were studied selectively using both primary and secondary antibody assay techniques and different cow's milk antigens. Techniques which demonstrate antibody–antigen binding revealed that the incidence, amount and immunoglobulin class of detectable antibody to bovine serum albumin (BSA) were not significantly different among IBD and control subjects. Only 13 of the 138 sera with the most anti-BSA by primary binding techniques had the capacity to precipitate spontaneously either BSA or antigens in raw (RSM) and pasteurized (PSM) skimmed milk. In passive haemagglutination studies, 41% of these 138 sera had the capacity to agglutinate BSA-coated erythrocytes, while the respective figures for RSM and PSM were 56% and 77%. Only in studies employing the passive haemagglutination of RSM-coated erythrocytes were high titres found more frequently in sera from patients with IBD than in sera from control subjects. Taken as a whole, this study fails to provide evidence for the pathogenetic significance of milk antibodies in IBD.

INTRODUCTION

Circulating antibodies to cow's milk proteins have been implicated in the etiology and pathogenesis of certain gastrointestinal (Taylor, Truelove & Wright, 1964; Heiner, Wilson & Lahey, 1964) and respiratory (Heiner, Sears & Kniker, 1962; Holland *et al.*, 1962; Heiner *et al.*, 1964) diseases. The relationship between these antibodies and disease has been difficult to establish since they frequently have been demonstrated in sera from healthy persons without symptoms upon ingesting milk and milk products (Lippard, Schloss & Johnson, 1936; Peterson & Good, 1963; Saperstein *et al.*, 1963; Rothberg & Farr, 1965; Kraft *et al.*, 1967), and since immunization to ingested proteins is a normal phenomenon in

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infants (Rothberg, 1969; Kletter *et al.*, 1971). In addition, among studies of patients with digestive disturbances such as ulcerative colitis, there have been conflicting reports of the incidence of high-titred serologic reactions to one or more cow's milk antigens (Taylor & Truelove, 1961; Sewell *et al.*, 1963, Dudek, Spiro & Thayer, 1965). The explanation for this conflict is unclear, but may relate to the antibody assay techniques employed.

Antibody assay techniques may be classified according to whether they measure primary antibody–antigen interactions (primary binding tests) or detect secondary manifestations of this interaction (Minden & Farr, 1969; 1971). Clinical responses mediated by this interaction might be considered tertiary manifestations (Minden & Farr, 1969). The primary binding tests are based on the principle of separating antibody-bound antigen from free antigen (Minden & Farr, 1969; 1971). For example, the quantitative separation of antibody–antigen complexes from free antigen may be accomplished by the addition of half-saturated ammonium sulphate (Farr, 1958). Secondary tests, however, are based upon manifestations of primary antibody–antigen interaction such as precipitation, agglutination or complement fixation, which may or may not follow this interaction (Minden & Farr, 1967; 1969; 1971). The separation of primary from secondary tests is more than of academic interest since the latter fail to identify certain antibody populations detectable by primary-binding techniques (Minden, Reid & Farr, 1966; Minden & Farr, 1967; 1969). Thus, primary-binding procedures should be used whenever possible in attempts to correlate immunological and clinical data.

The purpose of the present study was to learn if divergent results would be obtained by using both primary and secondary antibody assay techniques in an analysis of antibody to a cow's milk protein, bovine serum albumin (BSA), in sera from subjects with and without nonspecific inflammatory bowel disease (IBD). In addition, selected sera containing anti-BSA were characterized for antibodies to other cow's milk antigens.

MATERIALS AND METHODS

Subjects

Serum specimens were obtained from 760 subjects, ranging in age from 11 to 83 years. Patients with IBD (530) had either nonspecific ulcerative colitis (335) or Crohn's disease (195), i.e. regional enteritis (115) or ileocolitis-granulomatous colitis (80). Since there appears to be no relation between the presence of detectable anti-BSA and disease activity, duration and therapy in IBD (Kraft *et al.*, 1971), these patients were not subdivided according to such clinical factors. The 'control' group of 230 subjects included 137 individuals without demonstrable organic disease and 93 subjects with miscellaneous acute or chronic diseases not directly involving the gastrointestinal mucosa. No subjects with a primary dysgammaglobulinaemia were included in the IBD or control groups.

Antigens

Crystallized bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Company. Raw skimmed milk (RSM; kindly supplied by Borden and Co.) was lyophilized, stored at $-4°C$, and used after reconstitution to its original concentration. Commercially available fresh pasteurized skimmed milk (PSM) was prepared, stored and utilized in a similar manner.

Detection and quantification of circulating anti-BSA with the ammonium sulphate technique

Each serum sample was studied in duplicate for anti-BSA using a previously described modification (Rothberg, Kraft & Farr, 1967) of the ammonium sulphate technique (Farr, 1958). Results are expressed as the percentage of the antigen added (0·02 μg I*BSA nitrogen (N)) specifically bound by the test serum. Anti-BSA activity was considered present when the amount of I*BSA bound by a 1 : 5 dilution of the serum was 5% greater than the amount bound by duplicate samples with the I*BSA binding activity inhibited by the prior addition of unlabelled BSA (McCleery, Kraft & Rothberg, 1970). An antigen binding capacity (ABC-33) at 0·02 μg I*BSA N was determined for all sera which bound more than 33% of the antigen added. The experimental procedures and calculations have been described previously (Farr, 1958; Minden & Farr, 1967).

Indirect radio-immunodiffusion

The immunoglobulin classes associated with anti-BSA activity were assessed qualitatively in a 1 : 5 dilution of serum by indirect radio-immunodiffusion (Minden, Grey & Farr, 1967). Goat antisera against human γ, α or μ chains were obtained from Melpar, Inc. (Falls Church, Va.). The specificity of the observed binding was established by control experiments where unlabelled BSA (80 mg/ml) was added to the central well 24-hr prior to the addition of I*BSA.

Passive haemagglutination

The capacity of serum to agglutinate tanned, antigen-coated sheep erythrocytes was studied by the micro-method of Sever (1962) employing two-fold serial dilutions. The following predetermined optimal concentrations of antigen were employed: BSA, 0·75 mg/ml; RSM, 0·35 mg/ml; PSM, 0·35 mg/ml. The final washing and resuspension of the coated erythrocytes were performed in decomplemented 1 : 100 normal rabbit serum pre-absorbed with sheep erythrocytes (Minden *et al.*, 1966). All sera were tested to one of the antigens on the same day with the same preparation of antigen-coated cells. Antibody specificity was established by inhibiting positive reactions with 2 mg/ml of the appropriate antigen prior to adding the antigen-coated erythrocytes. Sera with titres of 1 : 16 or greater were considered to have antibody activity because: (a) in a preliminary BSA-haemagglutination study of 12 human sera without detectable anti-BSA by the ammonium sulphate technique, a maximum titre of 1 : 8 was obtained; and (b) the passive haemagglutination reactions to all three antigens could be inhibited at least to a titre of 1 : 8 or less.

Antigen precipitating capacity

The capacity of serum to precipitate I*BSA spontaneously was determined in duplicate using a variation of the technique described by Talmage & Maurer (1953). Specifically, 0·02 μg I*BSA N in 0·5 ml of 1 : 100 normal human serum-borate buffer diluent was added to 1·0-ml aliquots of 1 : 10 and 1 : 20 test serum dilutions. The tubes were incubated at 4°C for 6 days and agitated daily. Precipitating anti-BSA was considered detectable when a given serum precipitated more than 10% of the antigen added (Minden *et al.*, 1966).

Gel diffusion

A micro-Ouchterlony technique was employed. A 1-mm layer of special Noble agar (Difco Control No. 442–487) was applied to microscope slides and the central wells were

filled with undiluted serum samples. Six peripheral wells were filled with both RSM and PSM (undiluted, 1:2 and 1:4) and BSA (1 mg/ml), and most sera also were reacted with BSA in nine concentrations ranging from 10 to 0·05 mg/ml. In addition, tests for anti-BSA involved reacting six two-fold serial dilutions of selected sera in the peripheral wells against BSA 1 mg/ml or undiluted RSM in the central well. The slides were incubated for 48 hr at 4°C, washed for 48 hr in multiple changes of normal saline, dried and the protein precipitin lines stained with 0·6% Amido Black.

TABLE 1. A comparison of the incidence of detectable serum anti-bovine serum albumin (anti-BSA) among adults with inflammatory bowel disease and control subjects as determined with the ammonium sulphate technique

Group	Ages					
	21–40-year old			41+-year old		
	No. of subjects	No. with anti-BSA	% with anti-BSA	No. of subjects	No. with anti-BSA	% with anti-BSA
Ulcerative colitis	134	61	46	133	37	28
Crohn's disease						
Regional enteritis	51	26	51	38	6	16
Ileocolitis-granulomatous colitis	35	24	69	15	3	20
Control						
Healthy	42	14	33	95	18	19
Miscellaneous diseases*	28	11	39	65	11	17

* Diseases not directly involving the gastrointestinal mucosa.

RESULTS

Primary binding techniques

The incidence of detectable anti-BSA among adult patients with IBD and the control subjects was determined by the ammonium sulphate technique and the data are shown in Table 1. The only statistically significant difference was the higher incidence of detectable anti-BSA among 21–40-year old patients with ileocolitis or granulomatous colitis in contrast to the corresponding control groups ($P<0·02$). This observation probably is related to the finding that the mean age of this disease group was considerably younger than that of the 21–40-year old control subjects (Rothberg & Farr, 1965; Kraft *et al.*, 1967). Since the incidence of anti-BSA between the two control groups did not differ significantly, in subsequent studies they have been considered as a single group.

To learn if there might be a relationship between the *quantity* of circulating anti-BSA and the diagnoses under consideration, the incidence of sera containing large amounts of anti-BSA by the ammonium sulphate technique was determined. The sera from 122 subjects had sufficient anti-BSA to calculate an antigen-binding capacity. The ABC-33 values varied from 0·07 to 3·23 μg BSA N/ml. The incidences were similar among the four groups: ulcerative colitis, 12% of 335 patients; regional enteritis, 16% of 115; ileocolitis-granuloma-

tous colitis, 10% of 80; and controls, 7% of 230. Further, six of fifteen sera which bound more than 0·50 μg I*BSA N/ml were from control subjects. Of the remaining nine individuals, seven had ulcerative colitis and two had Crohn's disease.

The immunoglobulin classes associated with the anti-BSA activity were determined by indirect radio-immunodiffusion using all sera from both the IBD and control groups which bound more than 20% of 0·02 μg I*BSA N. Among these 138 sera, circulating anti-BSA predominantly was found associated with IgG alone (45%) or with IgG as well as with IgA (31%). In contrast to earlier data (Minden *et al.*, 1966; Kraft *et al.*, 1967; Korenblat *et al.*, 1968), eleven subjects (8%) were noted to have anti-BSA associated only with IgA. The less sensitive indirect radio-immunodiffusion technique (Minden, Anthony & Farr, 1969) failed to detect the anti-BSA in twenty-two sera (16%). The immunoglobulin classes associated with anti-BSA were very similar among the control and IBD groups.

Secondary manifestation techniques

The similarity of the results of the primary binding tests among IBD and control subjects does not preclude different findings when utilizing secondary antibody assay techniques. Accordingly, comparative studies were performed on the 138 sera which bound at least 20% of 0·02 μg I*BSA N. Sixty-seven of these subjects had ulcerative colitis, forty-six had Crohn's disease and twenty-five were control subjects. There was no correlation between the finding of anti-BSA by the ammonium sulphate technique and the finding of anti-BSA by the passive haemagglutination of BSA-coated erythrocytes. Eighty-one (59%) of these sera did not have detectable anti-BSA by the haemagglutination procedure (Fig. 1). Also, some sera which bound small amounts of I*BSA had high haemagglutination titres and haemagglutinating anti-BSA could not be detected in some sera with high BSA binding capacities. In addition, there was no relationship between the presence of IBD and a detectable capacity of sera to agglutinate BSA-coated erythrocytes. Indeed, this capacity was noted in a greater percentage of control subjects (56%) than those with ulcerative colitis (39%) or Crohn's disease (37%). Finally, six of the twelve individuals with haemagglutination titres of 1:512 or above were control subjects.

Precipitating antibody tests have been used extensively in attempts to correlate circulating antibody with disease. Using a sensitive precipitating assay for anti-BSA (Talmage & Maurer, 1953), only seven of the 138 sera had a positive response. The data from these seven subjects, two of whom were healthy controls, are presented in Table 2. In general, the capacity to make detectable precipitating antibody did not correlate with age or diagnosis and there was no direct relationship between the presence and amount of anti-BSA as determined by precipitation, ammonium sulphate or passive haemagglutination techniques. None of these seven patients had detectable precipitating anti-BSA when their sera were studied by the less sensitive gel diffusion technique. Only two subjects (L.T. and R.R.) made predominantly precipitating anti-BSA and these two sera failed to agglutinate BSA-coated erythrocytes.

To learn the relationship between the presence of readily detectable anti-BSA as determined with the ammonium sulphate test and antibodies to other cow's milk antigens, the same 138 sera were tested for haemagglutinating antibodies to RSM and PSM, and for precipitating antibodies to RSM and PSM. The divergent results obtained in tests for haemagglutinating antibodies to BSA and to these two milk antigen preparations are presented in Fig. 1. The use of the different antigens gave different values for the incidence

TABLE 2. Influence of different assay techniques in detecting circulating anti-bovine serum albumin (anti-BSA) activity among subjects with detectable precipitating anti-BSA

Subject	Age	Diagnosis	ABC-33 at 0·02 μg I*BSA N	Spontaneous precipitation*	Gel diffusion	Passive haemagglutination
R.S.	23	Healthy	0·74	28·4	—	256
C.M.	15	Regional enteritis	0·78	26·1	—	32
L.T.	25	Ileocolitis	+(22·1)†	24·1	—	0
R.R.	50	Ulcerative colitis	+(21·5)	23·5	—	2
S.S.	23	Healthy	1·16	16·8	—	1024
P.Z.	20	Ulcerative colitis	0·78	12·4	—	1024
F.K.	45	Ulcerative colitis	0·71	10·7	—	512

* Expressed as the % of 0·02 μg I*BSA N spontaneously precipitated by a 1 : 5 dilution of test serum. Precipitating anti-BSA is considered present when a given serum precipitates more than 10% of the antigen added.

† Anti-BSA was present in amounts insufficient to calculate an antigen-binding capacity. The figure in parentheses is the % of 0·02 μg I*BSA N bound by a 1 : 5 dilution of test serum and is comparable to the % spontaneously precipitated since the same amount of antigen was used in both tests.

of anti-milk antibodies (41% BSA, 56% RSM and 77% PSM) among the same 138 sera. Despite more positive haemagglutination responses to RSM, 61 sera which contained anti-BSA as measured by a primary-binding technique were unable to cause agglutination of RSM-coated erythrocytes. Similar to the findings with BSA, there was no relationship between the presence of IBD and a detectable capacity of sera to agglutinate PSM-coated erythrocytes. However, 24% of the sera from both IBD groups had haemagglutinating titres of 1 : 512 or above to RSM, in contrast to only 4% for sera from control subjects.

Next, the 138 sera were studied for precipitating antibodies using a micro-Ouchterlony technique. Sera from six of these subjects, all of whom had IBD, showed distinct precipitin reactions to both RSM and PSM. No serum reacted in agar gel to BSA and none of the seven subjects shown in Table 2 were among the six with detectable precipitin reactions to RSM and PSM.

DISCUSSION

The present study failed to show any quantitative or qualitative differences between antibodies to a purified cow's milk protein, BSA, in the circulation of patients with IBD and those in control subjects. Similar to a study of other human sera (Minden *et al.*, 1966), the secondary antibody tests failed to detect some anti-BSA populations demonstrable with the primary tests. In addition, the varying capacities of the antibodies to cause haemagglutination and precipitation resulted in a lack of correlation between the presence and amount of antibody as determined with the ammonium sulphate procedure and with the secondary assay techniques (Fig. 1 and Table 2). There also was no correlation between the results obtained using the passive haemagglutination and spontaneous precipitation assays. The variables which control whether secondary manifestations will occur following the primary interaction include (a) the quantities and physicochemical properties of both the

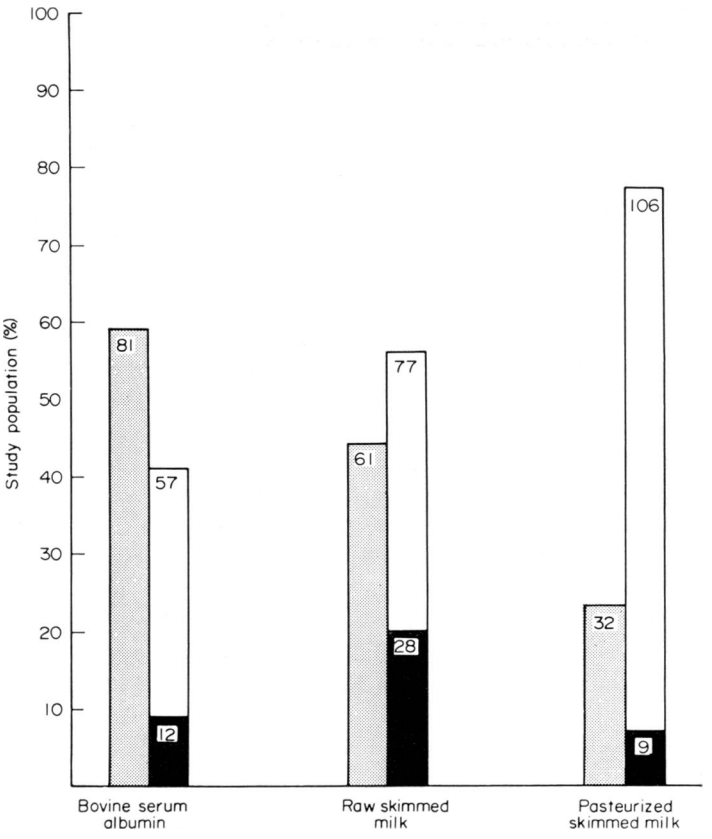

FIG. 1. Passive haemagglutination of cow's milk antigen-coated erythrocytes by 138 sera from both the inflammatory bowel disease and control groups which bound more than 20% of 0·02 μg I*BSA N. Stippled bar, negative; open bar, positive; solid bar, 1:512 or above.

antigen and antibody; (b) the average antigen-binding strength of the antibody populations present; and (c) the presence or absence of cofactors such as complement (Minden *et al.*, 1969; Minden & Farr, 1969; 1971).

Since primary-binding techniques are not applicable to studies of antibodies to mixtures such as RSM and PSM, secondary tests were used. Despite the use of only sera with positive BSA-binding capacities, only one of the multiple combinations of antibody assay techniques and antigen preparations used showed differences in the amount of antibodies to cow's milk proteins in patients with IBD in contrast to control subjects. Thus, 24% of subjects from both the ulcerative colitis and Crohn's disease groups had haemagglutination titres of 1:512 or greater when using RSM-coated erythrocytes, in contrast to only 4% of the control subjects. The failure to differentiate between ulcerative colitis and Crohn's disease suggests that the present data using RSM-coated erythrocytes should not be considered supportive of previous haemagglutination studies of adult patients with ulcerative colitis using casein or β-lactoglobulin. These earlier studies showed a relative excess of high titre haemagglutinating antibodies in sera from such patients (Taylor & Truelove, 1961, Taylor

et al., 1964); whereas sera from adults with regional enteritis demonstrated a distribution of these anti-milk antibodies similar to that found in healthy control subjects (Taylor *et al.*, 1964). In addition, other investigators also measured haemagglutinating antibodies to casein and β-lactoglobulin and could not distinguish between the serum antibody titres of patients with ulcerative colitis and those of healthy control subjects (Sewell *et al.*, 1963; Dudek *et al.*, 1965). The meaning of an increased incidence of high titre antisera to heterogeneous milk antigen preparations such as RSM and casein (Martinez-Resa *et al.*, 1968) in patients with ulcerative colitis remains unclear. Of the 138 subjects who were shown to be immunized to a purified milk protein, BSA, sixty-seven had ulcerative colitis and the sera from thirty-four of these patients failed to agglutinate RSM-coated erythrocytes. These findings suggest either that antibodies to milk play no pathogenetic role in IBD or, at most, that certain of these patients may demonstrate an increased permeability and reactivity of the intestinal mucosa to dietary antigens.

This study is consistent with what is known about oral immunization, the physiologic process by which humans become immunized to cow's milk proteins (Rothberg, 1969; Kletter *et al.*, 1971), and confirms the relatively poor precipitating capacity of human antibodies to cow's milk proteins. In addition, the data support the recent contention that most of the precipitating anti-milk antibodies found are not directed to the BSA fraction (Shapira *et al.*, 1971). The six sera which gave immunodiffusion lines when reacted in agar gel with either RSM or PSM did not spontaneously precipitate BSA in either of the tests used. In this regard, it is of interest that a high incidence of precipitating antibodies to RSM has been demonstrated in predominantly paediatric patients with serum IgA deficiency (Buckley & Dees, 1969). Available data suggest that the major milk antigen involved may be bovine IgM (Leikola & Vyas, 1971; Tomasi & Katz, 1971).

Studies directly relating human illness to the presence of precipitating antibodies to milk constituents must be interpreted with caution. The meaning of the finding that the six sera with precipitating antibodies to both RSM and PSM were only from patients with IBD is unclear. These few patients represented a small percentage of the 111 sera from patients with IBD which were so tested. Earlier investigators also found precipitating antibodies to milk proteins in normal individuals (Peterson & Good, 1963), and milk precipitating substances have been detected both in stools from thirty-six of thirty-eight individuals with acute diarrhoea unrelated to milk allergy and in stools from seven of eleven asymptomatic breast fed infants (Davis *et al.*, 1970). In addition, the demonstration of antibody in enteric secretions or faecal extracts does not necessarily signify that the origin of this antibody is the lymphoid tissue of the gastrointestinal tract since the gut is the major catabolic pathway for serum γ-globulins (Schultze & Heremans, 1966), and the passive transudation of serum anti-BSA into the gastrointestinal tract has been documented in normal animals (McCleery *et al.*, 1970). Finally, nonimmunologic precipitin lines (Plaut & Keonil, 1969; Kraft, Rothberg & Kriebel, 1970; Brown, 1971) might form between enteric secretions and cow's milk proteins.

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