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Sialoproteinaemia: lack of correlation with inhibition of *in vitro* lymphoblastosis induced by phytohaemagglutinin or alloantigen

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SUMMARY

Elevation of serum-bound sialic acid concentration in different disease states fails to correlate significantly with suppressive serum actions in mixed allogeneic lymphocyte cultures or phytohaemagglutinin cultures. Heat-decomplemented serum from patients with abnormal levels of bound sialic acid was added to parallel cultures containing similar blood lymphocyte populations derived from normal humans. Wide fluctuations of the rate of incorporation of tritiated thymidine into nucleoprotein indicated presence of suppressive elements other than sialoprotein in the added serum components. Serum with rising sialyl concentration derived from patients with cancer showed slight tendency to augment mixed lymphocyte and phytohaemagglutinin responses. The findings suggest that the previously documented nonspecific suppressive action of serum sialoprotein on human host lymphoblastic response to neuraminidase-treated cancer cells represents a mechanism unique to that culture system rather than a manifestation of a general immunoregulatory function of serum sialoprotein.

INTRODUCTION

Numerous reports have documented an increase of serum sialoproteins in cancer (Bogden *et al.*, 1967; Gottschalk, 1960; Watkins *et al.*, 1974; Weimer *et al.*, 1957; Winzler, 1955; Zacharia & Pollard, 1969) and certain nonmalignant diseases (Gottschalk, 1960) of both animals and man. Of the natural sialic acids, only N-acetylneuraminic acid has been shown to occur in man (Martensson, Raal & Svennerholm, 1958). Apffel & Peters (1969, 1970) postulated that one biologic role of carbohydrate-rich sialic acid containing glycoproteins is the regulation of antigenic expression. According to their axiom, the carbohydrate moiety of serum glycoproteins is the biologically active part of the molecule concerned with repression of antigenic determinants, and this regulatory role is entirely nonspecific in nature.

We have previously reported the ability of sialoglycoproteins of both normal and cancer sera to attentuate the *in vitro* lymphoblastic transformation of host lymphocytes in response to *Vibrio cholerae* neuraminidase-treated tumour cells (Gray & Watkins, 1975; Watkins *et al.*, 1974). In that test system a direct relationship was evident between bound sialic acid concentration of serum added to cultures and the degree of lymphoblastic response. Furthermore, this blocking effect of sialoprotein could be abrogated by enzymatically releasing terminally situated sialic acid from sialoglycoproteins and removing it from the serum by dialysis. The blocking ability of sialoglycoproteins was entirely nonspecific not only for the type of tumour but even for the presence of malignant disease itself.

This study was undertaken to determine if a relationship existed between the concentration of serum sialoglycoproteins in a variety of different disease states and inhibition of *in vitro* lymphoblastic response of normal human lymphocytes to mitogenic and alloantigenic stimulation.

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MATERIALS AND METHODS

Lymphocytes. For phytohaemagglutinin (PHA)-stimulated cultures, lymphocytes were obtained from a healthy male laboratory worker. For mixed lymphocyte cultures (MLC), lymphocytes were obtained from two healthy male laboratory workers who had previously been shown to have strongly reactive MLC assays.

Peripheral venous blood was drawn into heparinized syringes, and lymphocytes were obtained by centrifugation in a Hypaque–Ficoll gradient (Thorsby & Bratlie, 1970). Cells procured by this method consisted of greater than 90% small lymphocytes, and viability as assessed by eosin exclusion consistently exceeded 95%.

Sera. Sera were obtained from five groups of patients. Group 1 consisted of twenty-one patients with a variety of common malignant tumours, including adenocarcinoma of the colon, rectum, pancreas and breast; epidermoid carcinoma of lung and mouth; glioblastoma of brain; Hodgkin's disease; and cloacogenic carcinoma of the anus. In group 2 were eleven patients with a variety of benign tumours of the parotid, thyroid, and parathyroid glands, and benign tumours of the colon, rectum and breast. Group 3 was composed of ten patients in terminal renal failure who were receiving extracorporeal dialysis. In group 4 were seven patients with advanced noncancerous liver disease, and in group 5 were nine patients with a variety of miscellaneous nonmalignant diseases. Before any operation, 20 ml of blood was collected from patients who were not receiving drug therapy. After clot retraction, the blood samples were centrifuged at 1500 *g* for 20 min, and the sera obtained were decomplemented by incubating at 56°C for 30 min. Serum samples were stored at −20°C until used. Storage at −20°C and repeated freezing and thawing do not significantly alter the concentration of bound sialic acid of serum samples or influence the ability of serum to support *in vitro* lymphocyte response (Watkins *et al.*, 1974).

Serum-bound sialic acid. Concentration of bound sialic acid in peripheral venous serum was measured by the thiobarbituric acid assay method measuring absorbance of 1 cm light path distance specimens at 549 nm and 532 nm in a Gilford Model 240 spectrophotometer (Warren, 1959).

Phytohaemagglutinin-stimulated cultures. PHA (type P, Difco, Control No. 551099) was used in a suboptimal stimulatory concentration of 2·5 μg ml^{-1} of the final tissue culture fluid. Optimal stimulatory PHA concentration was previously determined as being 15·0 μg ml^{-1}. Cultures were performed in Medium RPMI 1640 (Microbiological Associates) supplemented with 1% fresh-thawed glutamine and 20% decomplemented test serum and in microculture plates (Linbro, 1S-MRC-96) in a final volume of 0·22 ml per well. Cell concentration was either $0{\cdot}125\times10^6$ or $0{\cdot}5\times10^6$ lymphocytes per well. Cells, serum and PHA were distributed to the culture plates so that the lymphocytes were exposed to the test sera before being exposed to PHA. All cultures were performed in an atmosphere of 5% CO_2 in air at 37°C. Pulse tagging was performed on the fourth day of culture with 1 μCi of tritiated thymidine (New England Nuclear, specific activity 50·8 Ci/mmole) per well, and the cells were aspirated onto glass fibre strips after 17 hr, using a MASH-II automatic cell harvester (Microbiological Associates). Radioactivity incorporated into lymphocyte nucleoprotein was measured using conventional liquid scintillation techniques (Watkins *et al.*, 1974).

Mixed lymphocyte cultures. Two-way MLC were performed using $0{\cdot}5\times10^6$ cells of each lymphocyte type per well. Cultures were performed in Medium 1640 containing 1% fresh-thawed glutamine and 20% decomplemented test serum. Pulse tagging was performed on the fourth day of culture. Details of pulse tagging and culture conditions were identical to those described for PHA-stimulated cultures.

One-way MLC were performed using $0{\cdot}1\times10^6$ mitomycin and neuraminidase-treated stimulator cells and $0{\cdot}5\times10^6$ effector cells per well. Polynucleotide synthesis was blocked in stimulator cells by prior incubation with mitomycin-C (25 μg/10^7 cells/ml for 20 min). Stimulator lymphocytes were further incubated with *Vibrio cholerae* neuraminidase (Behringwerke) 100 EU/10^7 cells/ml in an acetate buffered saline solution, pH 7·2, for 1 hr to remove bound sialic acid from the cell surface. The method of mitomycin and neuraminidase treatment was identical to that used in previous experiments to treat cancer cells. In those experiments, a positive correlation was found between lymphoblastic response and the concentration of bound sialic acid in serum added to cultures (Watkins *et al.*, 1974).

All cultures were performed in triplicate, and the results were recorded as mean values of triplicate cultures. Mean serum-bound sialic acid concentrations of the various groups were compared using a two-tailed *t*-test. Correlation between serum-bound sialic acid and lymphoblastic response was assessed using linear regression and analysis of variance. *P* values of less than 0·05 were considered significant.

RESULTS

Serum-bound sialic acid

The previously determined mean serum-bound sialic acid concentration for a group of thirty-one normal subjects was $1{\cdot}69\pm0{\cdot}19$ (SD) μmol ml^{-1}. The concentration of serum-bound sialic acid (Table 1) was significantly elevated in the group of twenty-one patients with malignancy ($P<0{\cdot}001$) and was similar to that reported for malignancy in an earlier group of cancer patients (Watkins *et al.*, 1974). The group of ten patients with terminal renal failure also had significantly elevated serum sialic acid levels ($P<0{\cdot}02$) as did the group of patients with miscellaneous diseases ($P<0{\cdot}01$). Patients with benign tumours had serum-bound sialic acid levels that were not significantly different from normal ($P>0{\cdot}70$).

TABLE 1. Serum-bound sialic acid in various disease states

Diagnosis	No. in group	Bound sialic acid: micromoles ml^{-1} NANA+SD	Bound sialic acid: Range	*t*-test *P*: group *v.* normal subjects*
Malignant tumour	21	2·21±0·43	1·52–3·13	<0·001
Benign tumour	11	1·73±0·30	1·30–2·19	>0·70
Renal failure	10	2·13±0·43	1·71–3·23	<0·02
Liver disease (nonmalignant)	7	1·57±0·38	1·05–1·96	>0·40
Miscellaneous†	9	2·10±0·33	1·69–2·66	<0·01

* Thirty-one normal laboratory workers; mean concentration±s.d. 1·69±0·19 micromoles NANA ml^{-1}; range, 1·30–2·21.

† Includes one case each of von Willebrand's disease (1·69), Crohn's colitis (2·66), ulcerative colitis (1·95), cholelithiasis (1·91), breast adenitis (1·32), cholecystitis (2·42), inguinal hernia (1·86), duodenal ulcer (2·43) and lymph node hyperplasia (2·16).

Patients with advanced nonmalignant liver disease showed low serum-bound sialic acid levels, but the small group analyzed was not statistically different from the normal group ($P>0·40$).

PHA-stimulated culture responses

PHA-stimulated cultures were performed twice in each of the twenty-one cancer sera (two separate experiments). The mean and each individual cpm for stimulated cultures were then plotted against the concentration of serum sialic acid. On analysis of 126 separate data points, a significant correlation was shown between serum-bound sialic acid concentration and degree of lymphoblastic transformation ($P<0·005$), thus indicating an increase in serum-bound sialic acid correlated with an increase in lymphoblastic transformation. Correlation between serum-bound sialic acid concentration and degree of lymphoblastic response was not significant when lymphocytes were cultured in sera from patients with advanced nonmalignant liver disease (two experiments, $P>0·10$), benign tumours (one experiment, $P>0·05$), or miscellaneous disease states (one experiment, $P>0·50$). A significant correlation ($P<0·01$) was evident when the test sera were obtained from patients with advanced renal disease. However, if the one test serum with an unusually high bound sialic acid concentration was not included in the correlation analysis, the correlation became insignificant ($P>0·50$). However, the correlation obtained with sera from patients with renal failure was opposite to that obtained with cancer sera in which an increase in serum-bound sialic acid correlated with an increase in lymphoblastic response.

MLC responses

MLC were performed in each of the sera from the five groups of patients. No significant correlation was shown between serum-bound sialic acid concentration and degree of lymphoblastic response in any of the five groups tested. Although not at a statistically significant level, the trend seen in PHA-stimulated cultures was similarly observed in MLC assays. A positive correlative trend was seen between serum-bound sialic acid concentration and lymphoblastic response in sera from cancer-bearing patients, but a negative trend was seen when sera from patients with advanced non malignant liver disease, renal failure, or benign tumours were used in cultures. In cultures using sera from patients with miscellaneous disease states a nonsignificant positive trend was observed ($P>0·50$). As it appeared possible that the phenomenon of nonspecific blocking of lymphocyte response to antigenic sites was restricted to tissue antigens exposed by neuraminidase treatment, one-way MLC using neuraminidase-treated stimulator lymphocytes was performed. The response closely resembled that obtained in two-way MLC, and no significant correlation was shown ($P>0·10$).

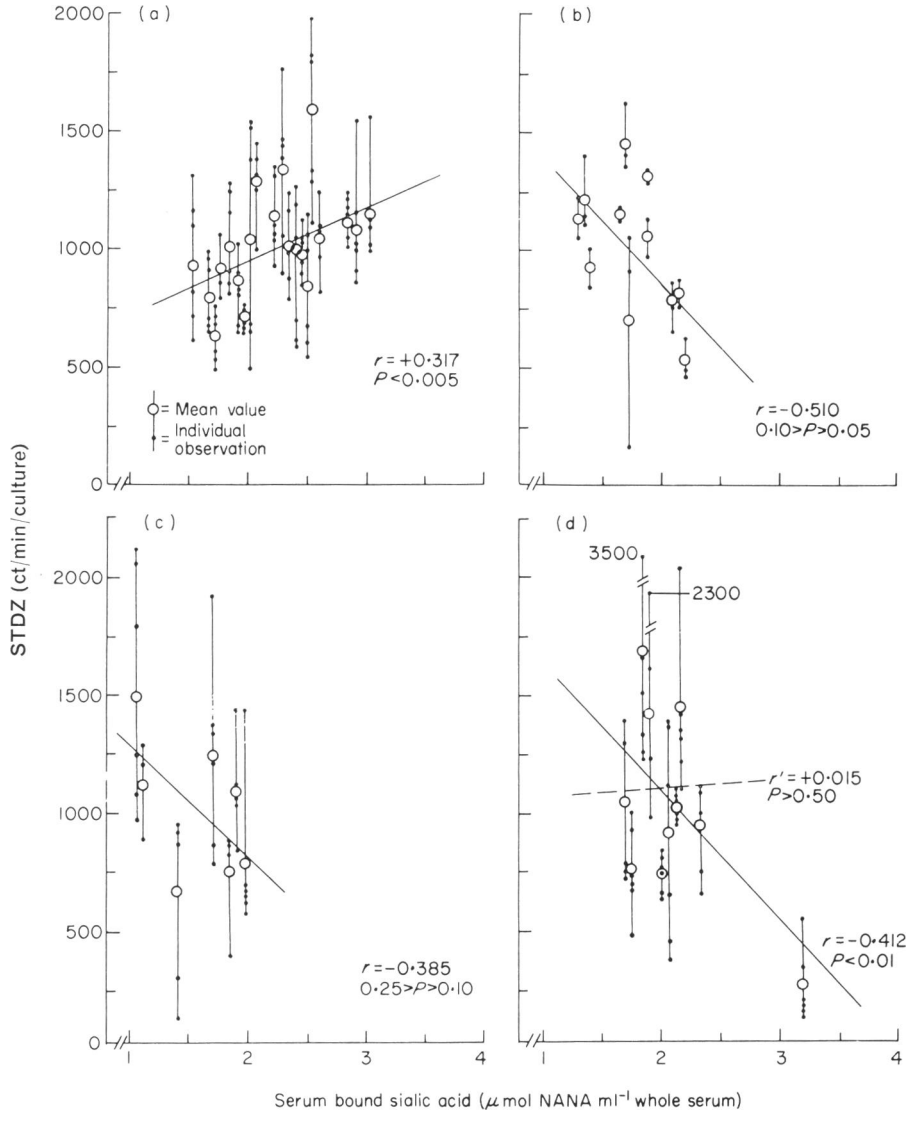

FIG. 1. Linear regression analysis for PHA-stimulated cultures of observed cpm and bound sialic acid concentration of sera used in cultures. Mean and individual cpm shown for each serum sample. Serum samples obtained from four patient groups: (a) patients with cancer, (b) patients bearing benign tumours, (c) patients with advanced liver disease, and (d) patients with renal failure. Significant negative correlation when cultures performed in renal insufficiency sera, although deletion of cultures performed in the one serum sample with very high bound sialic concentration renders correlation insignificant. STDZ ct/min = ct/min for individual cultures standardized to correct for ct/min variations between different experiments.

DISCUSSION

One of the many serum alterations occurring in cancer in animals (El-Ghaffar & Assad, 1967; Bogden *et al.*, 1967; Weimer *et al.*, 1957; Zacharia & Pollard, 1969) and in man (Ashikawa *et al.*, 1971) is a rise in certain alpha glycoproteins. A rise in serum-bound sialic acid has also been demonstrated to occur in cancer (McNeil *et al.*, 1965; Saifer & Gerstenfeld, 1962; Watkins *et al.*, 1974; Winzler, 1955) and in many other disease states (Winzler, 1955). Of the serum glycoproteins, the alpha glycoproteins have the highest content of sialic acid.

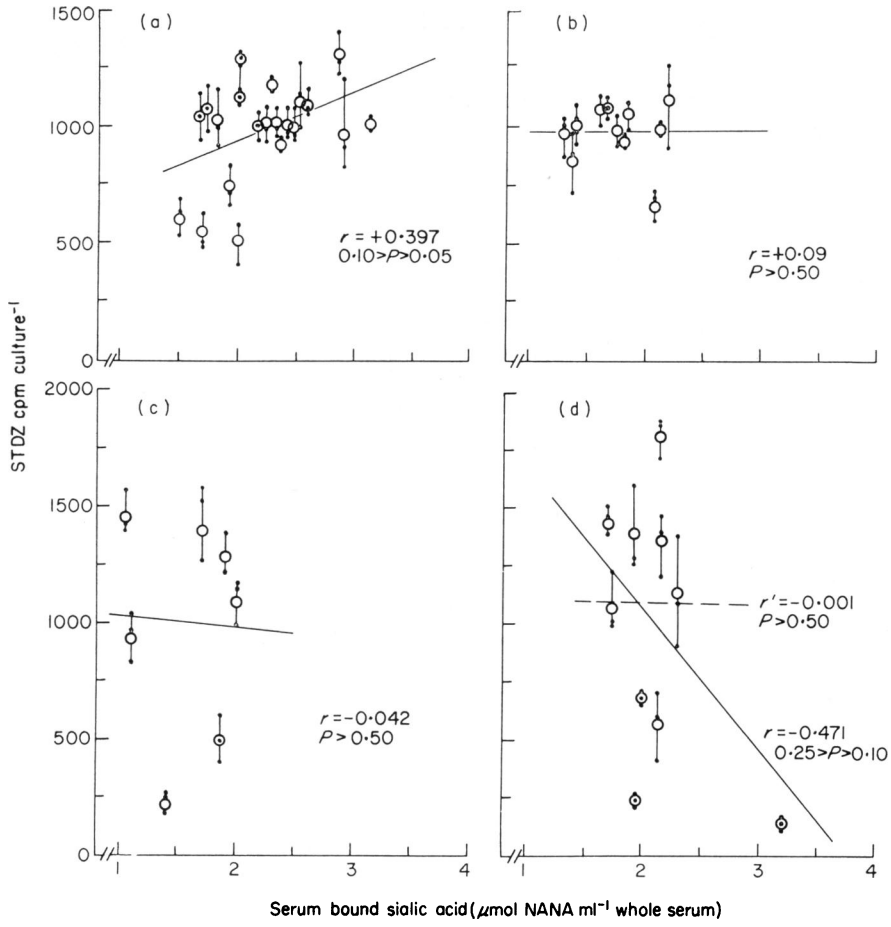

FIG. 2. Linear regression analysis, for two-way mixed lymphocyte cultures of observed cpm and bound sialic acid concentration of sera used in cultures. Mean and individual cpm shown for each serum sample. Serum samples obtained from four patient groups: (a) patients with cancer, (b) patients bearing benign tumours, (c) patients with advanced liver disease, and (d) patients with renal failure. No significant correlation was obtained in any patient group. STDZ cpm = cpm for individual cultures standardized to correct for cpm variations between different experiments.

Considerable evidence has shown that sera from cancer-bearing subjects are immunosuppressive in that they can inhibit the *in vitro* blastic transformation of lymphocytes to a variety of mitogens (Badger, Cooperband & Mannick, 1974; Horwitz, Normansell & Brooks, 1974; Hsu & LoGerfo, 1972) and to tissue alloantigens (Badger *et al.*, 1974; Parmely & Thompson, 1974). Normal human serum has also been shown to contain alphaglobulins which can suppress the *in vitro* immune response of lymphocytes to mitogens and specific antigens (Chase, 1972; Cooperband *et al.*, 1968). A suppressant role of alphaglobulins has also been demonstrated *in vivo* (Ashikawa *et al.*, 1971; Whang, Chun & Neter, 1969). Changing levels of alphaglycoproteins in guinea-pigs after administration of dinitrofluorobenzene has been correlated with suppression of lymphocyte response to a variety of common mitogens (Burger, Lilly & Vetto, 1974). The addition of crystalline N-acetylneuraminic acid (NANA) to *in vitro* cultures has been reported as suppressing lymphocyte response to PHA, varidase, and allogeneic cells (Han, 1975). (Unreported observations in our laboratory on PHA-stimulated cultures and in MLC do not support this latter finding with up to 25 μm of crystalline NANA ml^{-1}.) By testing purified human serum proteins separately, Johannsen, Carlsson & Heide (1974) did not find any correlation between suppressant activity and carbohydrate content, although several alphaglycoproteins were suppressive to

both mitogen and alloantigen-induced transformation. A positive correlation between plasma inhibitory effect and alphaglycoprotein concentration has been reported in PHA-stimulated cultures (Hsu & LoGerfo, 1972). The serum inhibitory effect has also been correlated with alphafetoprotein levels in hepatoma-bearing rats (Parmely & Thompson, 1974). Horwitz *et al.* (1974) correlated inhibitory activity of fractionated cancer sera with IgG-rich fractions. A nonspecific immunosuppressive peptide fraction having suppressive properties both *in vitro* and *in vivo* has been isolated from sera of cancer patients (Glasgow *et al.*, 1974; Nimberg *et al.*, 1975). The fraction is reported to be free of neutral sugars and sialic acid.

Mitogen-induced lymphocyte transformation has been shown to be inhibited by sera or plasma from patients with uraemia (Silk, 1967), alcoholic liver cirrhosis (Hsu & Leevy, 1971), and acute and chronic hepatitis (Wands, Perrotto & Isselbacher, 1975).

We have previously reported a strong correlation between serum-bound sialic acid of cancer sera and lymphocyte transformation response to neuraminidase-treated tumour cells *in vitro* (Gray & Watkins, 1975; Watkins & Gray, 1974; Watkins *et al.*, 1974). The experiments reported here were designed to determine if the hypothesis of Apffel & Peters (1969, 1970) of nonspecific immuno suppression by sialoglycoproteins held true for mitogen and alloantigen-induced lymphocyte transformation using sera from several groups of patients previously shown to be suppressive. The results indicate that no such correlation exists for patients with cancer, benign tumours, or advanced renal or liver disease. In PHA-stimulated cultures, a significant correlation was shown opposite to that expected if, in fact, suppression were mediated by serum sialoglycoproteins. No correlation was evident in the group of patients with a variety of miscellaneous non-neoplastic disorders, although it is impossible to draw any further conclusions as several different disease states are represented.

Having previously demonstrated a significant suppressive role of serum sialoglycoproteins *in vitro*, we consider that several explanations are possible for these findings. As a suboptimal dose of PHA was used in all cultures, it is highly unlikely that excessive PHA was responsible for overriding any weak inhibitory effect that might otherwise have been observed. As absolute ct/min in parallel cultures containing the same types of cells and mitogens varied widely between different sera, some undetermined serum factor or factors, other than serum-bound sialic acid, were obviously responsible for the differences in activity between cultures containing different sera. It is unlikely that a previously observed correlation between serum sialic acid and blocking of lymphocyte transformation was an artifact as it was statistically significant, and enzymatic removal of sialic acid from serum sialoglycoproteins also removed the inhibitory effect. It appears most likely that the phenomenon of sialoglycoprotein-mediated suppression of lymphoblastic transformation only occurs when lymphocytes are responding to tumour-associated antigens. As the serum changes that occur in advanced renal and liver disease are complex, several other factors may be present and capable of causing a blocking effect. Although the number of samples in each group was small, a tendency toward a serum sialic acid blocking effect in both PHA-stimulated and MLC assays was evident. However, the large difference in observed ct/min for different serum cultures within each group again suggests other undetermined factors as being of greater importance in determining the degree of lymphoblastic response.

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