Clin. exp. Immunol. (1971) **9**, 45–56.

DELAYED CUTANEOUS HYPERSENSITIVITY REACTIONS TO MEMBRANE EXTRACTS OF HUMAN TUMOUR CELLS*

M. E. OREN† AND R. B. HERBERMAN

Immunology Branch, National Cancer Institute, Bethesda, Maryland

(*Received* 10 *November* 1970)

SUMMARY

Autochthonous tumour cell membrane extracts produced delayed hypersensitivity reactions in patients with a variety of neoplastic diseases. When tested at low protein concentrations ($\leqslant$0·33 mg/0·1 ml), autochthonous tumour extracts produced positive reactions in 50% of non-anergic cancer patients. The same dose of autochthonous leucocyte membranes produced a significantly lower incidence of positive reactions (10%) in normal volunteers. In contrast, the cancer patients were less reactive than the normal volunteers to a battery of standard skin test antigens. In patients with acute lymphocytic leukaemia, positive reactions were more often obtained during remission. The protein concentration of the extracts was found to be an important factor in these studies. Extracts with high protein concentrations elicited positive reactions in both the patients and in the normal volunteers. It remains to be determined whether the reactions to the high protein concentrations are due to non-specific inflammation or to a low level of autoimmunity in both patients and controls.

INTRODUCTION

There is considerable evidence for the important role of cellular immunity in the rejection of allografts and of tumour transplants (Hellström & Möller, 1965). In several studies with experimental animal systems, delayed hypersensitivity reactions have provided evidence for cellular immunity to alloantigens and to tumour-specific antigens (Brent, Brown & Medawar, 1958; Kahan, 1967; Churchill *et al.*, 1968). Similar delayed skin reactions to human tumour cell antigens have been reported. Hughes & Lytton (1964) and Stewart (1969a, b)

* A preliminary communication was presented at the Xth International Cancer Congress, 22–29 May 1970, Houston, Texas, U.S.A.

† Present address: Department of Internal Medicine, Yale–New Haven Hospital, New Haven, Connecticut, U.S.A.

Correspondence: Dr Ronald B. Herberman, National Institutes of Health, National Cancer Institute, GL&C, Building 10, Room 4B18, 9000 Rockville Pike, Bethesda, Maryland 20014, U.S.A.

performed intradermal skin tests on patients with various carcinomas. Delayed-type hypersensitivity reactions were elicited in about 25% by autochthonous cell-free tumour extracts. We have previously reported (Herberman & Oren, 1969; Oren & Herberman, 1970), in preliminary fashion, success in eliciting delayed hypersensitivity reactions to autochthonous membrane extracts in 50% of non-anergic patients with leukaemia, lymphoma and carcinoma. Some patients with Burkitt's lymphoma (Fass, Herberman & Ziegler, 1970a) and some with malignant melanoma (Fass *et al.*, 1970b) had detectable delayed hypersensitivity reactions to similar extracts containing the tumour cell membranes. The skin reactivity observed in the studies of Fass *et al.* (1970a, b) was related to the clinical status of the patients. In patients with Burkitt's lymphoma (Fass *et al.*, 1970a), positive reactions were usually obtained in remission only and positivity was correlated with sustained remission. With malignant melanoma, positive reactions were obtained only in patients with localized disease (Fass *et al.*, 1970b).

In the present report, results of skin testing with membrane extracts of autochthonous tumour cells in patients with leukaemia, lymphoma and carcinoma are presented in detail. Results of control studies, in which normal volunteers were tested with membrane extracts of their autochthonous cells, are also presented.

MATERIALS AND METHODS

Subjects studied

One hundred and five sets of skin tests were carried out on seventy-six patients with a variety of leukaemias and other tumours, and on twenty-nine normal volunteers. Patients admitted to the Clinical Center of the National Institutes of Health were selected on the basis of availability of tumour tissue and clinical fitness to participate. The procedure was carefully explained to each subject and informed consent was obtained.

Skin tests were also performed on twenty-five normal adult volunteers, 25–46 years of age, and on four siblings of paediatric patients, 3–9 years old.

Preparation of membrane extracts

Sterile technique was used for all procedures. Membrane extracts of tumour cells from biopsies, leucocytes from leucaphereses, and control tissues when available were prepared by freeze-thawing and low ionic strength extraction (Table 1), a modification of the methods of Davies (1966) and Mann *et al.* (1968). Extracts prepared in this manner have been shown to be rich in transplantation and tumour antigens (Davies, 1966; Mann *et al.*, 1968). A crude membrane pellet was obtained from the pooled extracts by centrifugation at 105,000 *g* for 1 hr and the membranes resuspended in normal saline.

Protein concentration was measured by the method of Lowry, Rosebrough & Farr (1952). Dilutions of the membranes in saline were then prepared, to cover a broad range of protein concentrations. Sterility of the final preparations was tested by culture on blood agar plates and in thioglycollate broth. Only sterile extracts were used for skin testing.

Extracts prepared in this manner contained cell-surface membranes (Davies, 1966). The presence of surface membranes was confirmed in all of our initial preparations by the ability to absorb anti-HL-A antibody activity from a variety of leucocyte alloantisera, and in some preparations by electron microscopy. Initial studies indicated that cutaneous reactivity was elicited only by the membrane fraction, which was sedimented at 105,000 *g* for 60 min. The

concentrated 105,000 *g* supernatant, containing some cell sap, elicited little or no skin reactivity.

In one of the control studies with normal volunteers, individual cell types were separated from the peripheral blood. The lymphocytes were purified by passage of buffy coat rich heparinized plasma through a nylon fibre column (Greenwalt, Gajewski & McKenna, 1962). Granulocytes were separated from buffy coat rich plasma by sedimentation at 45 *g* for 10 min. Differential counts showed that the preparations contained greater than 90% of the particular cell type. Erythrocytes were obtained from blood that was centrifuged at 400 *g* for 3 min. Most of the leucocytes remained in the supernatant plasma, and the erythrocytes were taken from a portion of the sediment that was some distance from the buffy coat. After four washes, the percentage of contaminating leucocytes was less than 0·1%.

TABLE 1. Preparation of membrane extracts

1. Freeze-thaw cells
2. Centrifuge at 500 *g* for 10 min
3. Repeated extraction* with 0·14 M NaCl
4. Extraction* with 0·07 M NaCl (× 2)
5. Extraction with 0·035 M NaCl (× 2)
6. Crude membrane pellet obtained from pooled supernatant fluids by centrifugation at 105,000 *g* for 1 hr
7. Suspension of membranes in 0·14 M NaCl

* Extraction consisted of suspension in saline, incubation at 4°C for 10 min, and centrifugation at 500 *g* for 10 min.

Administration and interpretation of skin tests

Skin tests were performed by intradermal inoculation of 0·1 ml of the membrane preparations, usually on the subject's back. At the same time, to assess the ability of the recipients to manifest a delayed skin reaction, tests were performed with 0·1 ml of each of the following standard skin test antigens: candida (dermatophytin '0' 1:100, Hollister-Stier Laboratories, Spokane, Washington), histoplasmin (Parke Davis and Company, Detroit, Michigan); mumps antigen (Eli Lilly and Company, Indianapolis, Indiana); trichophyton (dermatophytin 1:30, Hollister-Stier Laboratories, Spokane, Washington); and tuberculin (intermediate strength purified protein derivative of tuberculin, 0·0002 mg of PPD, Parke Davis and Company, Detroit, Michigan). Some of the patients were also sensitized with dinitrochlorobenzene (DNCB, Brown *et al.*, 1967).

Skin tests were observed at 15 min, 2 hr, 24 hr and 48 hr after inoculation and measurements made with a vernier caliper. The reactions were usually read by the author who had not done the inoculations, without knowledge of the nature of the material injected at each site. Immediate weal-and-flare reactions of 15–30 min duration were frequently seen, even in anergic patients. These reactions were not closely associated with the development of delayed hypersensitivity. The delayed reactions were maximal at 24–48 hr. Induration of 5 mm or greater at 48 hr was considered a positive skin test. If the patient did not react to any

of the standard antigens (nor to DNCB sensitization if performed), he was considered anergic. More than eighty skin punch biopsies of positive and negative reactions were carried out and evaluated by one of us (R.H.). The slides were coded and the evaluation was performed without knowledge of which slides represented tests with tumour extract or control extracts, or of how the tests were interpreted clinically. In addition, many of the slides were reviewed with Dr Byron Waksman of Yale University. There was generally very good agreement between clinical readings and biopsies as to typicality for delayed hypersensitivity.

RESULTS

Delayed cutaneous reactivity to bacterial and viral antigens

Reactions to skin tests with a group of standard bacterial and viral antigens are shown in Table 2. Differences were noted between patients as a group and normal volunteers in responsiveness to the five standard antigens. 21% of the cancer patients were anergic. Among the non-anergic patients, the mode response was to one of the five antigens; 83·4% responded to only one or two antigens. In contrast none of the normal volunteers were anergic; 51·7% responded to three or more of the five antigens. The modal number of antigens responded to was two to three in the control group. Differences in responsiveness to individual antigens are shown in Table 3.

TABLE 2. Responsiveness to skin testing with standard antigens

	No. of standard tests responded to (of five)					
	1	2	3	4	5	0 (Anergic)
	(No. and % non-anergic patients responding)					No. and % total patients tested
Patients						
< 15 years old	7 (70)	2 (20)	1 (10)	—	—	1 (9)
> 15 years old	21 (42)	20 (40)	6 (12)	3 (6)	—	15 (23·1)
Total	28 (46·7)	22 (36·7)	7 (11·7)	3 (5·0)	—	16 (21·1)
Normal volunteers						
< 15 years old	2 (50)	2 (50)	—	—	—	0 (0)
> 15 years old	2 (7·0)	8 (32·0)	10 (40·0)	4 (16·0)	1 (4·0)	0 (0)
Total	4 (13·8)	10 (34·5)	10 (34·5)	4 (13·8)	1 (3·4)	0 (0)

Skin testing with autochthonous tumour extract

Table 4 gives the number of patients in each disease category that was tested. Only the non-anergic subjects, according to the definition given above, were considered suitable for evaluation. Forty-two of the sixty non-anergic patients (70%) gave positive reactions to at least one of the concentrations of autochthonous tumour membranes (Table 4). Patients with

TABLE 3. Responses to skin testing with standard antigens (non-anergic patients)

Antigen	Candida			Histoplasmin			Mumps			PPD			Trichophyton			DNCB		
	+	−	%+	+	−	%+	+	−	%+	+	−	%+	+	−	%+	+	−	%+
Patients																		
<15 years old	1	5	17	1	6	14	5	2	71·5	0	8	0	0	6	0	4	1	80
>15 years old	17	29	40	13	35	37·1	40	8	83·3	10	36	21·7	7	27	20·6	6	1	85·7
Total	18	34	34·6	14	41	25·5	45	10	81·8	10	44	22·7	7	33	17·5	10	2	83·3
Normal volunteers																		
<15 years old	3	1	75	0	4	0	3	1	75	0	4	0	0	4	0			
>15 years old	18	6	75	5	18	27·8	24	0	100	8	15	34·8	10	14	71·4	1	0	100
Total	21	7	75	5	22	18·5	27	1	96·4	8	19	29·6	10	18	55·6	1	0	100

acute myelocytic leukaemia, lymphoma, paraproteinaemia and carcinoma gave high response rates, whereas patients with lymphocytic leukaemia and chronic myelocytic leukaemia were less reactive. Biopsies of positive reactions showed lymphocytic and histiocytic perivascular infiltrates in the upper dermis, compatible with delayed hypersensitivity reactions. Biopsies of negative reactions showed either minimal cellular response or polymorphonuclear cell infiltrates.

Five patients with leukaemia were tested simultaneously with up to 5×10^7 freshly harvested, intact tumour cells and with membrane extracts. In three of these patients, positive reactions were produced by the intact cells as well as by the extracts. The other two patients had negative reactions to both whole cells and extracts.

The reaction of four other patients with leukaemia to cell membranes isolated by other procedures (McCollester & Semente, 1966; Warren, Glick & Nass, 1967; Boone *et al.*, 1969) was similar to that obtained with the usual extract.

TABLE 4. Responses to skin testing with autologous tumour and normal tissue membrane extracts I: tested at any concentration of antigen protein

Disease	No. tested	No. not anergic	No. positive reactions (%)
Acute lymphocytic leukaemia	16	15	7 (47)
Chronic lymphocytic leukaemia	16	14	7 (50)
Lymphoma, Hodgkin's disease	8	6	5 (83)
Mycosis fungoides	4	1	0 (0)
Acute myelocytic leukaemia	15	13	12 (92)
Chronic myelocytic leukaemia	15	12	7 (58)
Gammopathies (multiple myeloma, Waldenström's macroglobulinaemia)	5	4	3 (75)
Carcinoma, melanoma, Wilm's tumour	8	6	6 (100)
Total	87	71	47 (66)
Normal volunteers	29	29	22 (76)

Skin testing of controls with autochthonous extracts

Some of the non-leukaemic patients also gave positive reactions to membrane extracts of their normal peripheral blood leucocytes. To evaluate the nature of this reactivity twenty-five normal adult volunteers and four siblings of paediatric patients were skin tested with membrane extracts of autochthonous peripheral leucocytes. More than three-quarters of these control individuals responded with reactions typical in appearance, time course and histology for human delayed hypersensitivity (Table 4).

Effect of antigen protein concentration

When responses were tabulated according to the protein concentration of the antigen inoculated, it was found that most of the positive reactions to the normal leucocyte extracts, in both the non-leukaemic patients and in the controls, were elicited by preparations containing high protein concentrations. None of the patients and only about 10% of the

normal volunteers gave positive reactions to control autochthonous extracts at protein concentrations of 0·33 protein/0·1 ml or less (Table 5).

In view of these results with the normal volunteers, the patient data were reanalysed for the relationship of positive reactions to protein concentration. Forty-four of the patients had been tested with tumour membrane extracts at low protein concentrations, i.e. ⩽0·33 mg protein/0·1 ml (Table 5). Twenty-two of these patients (50%) gave positive reactions even at these concentrations. This degree of responsiveness is highly significantly different from that of the normal volunteers ($P<0·0003$, χ^2 analysis). The individual data for acute myelocytic leukaemic (AML) were also significantly different ($P<0·0003$, χ^2) from that of the normal volunteers. One patient with AML responded to a protein concentration of only 0·005 mg/0·1 ml. The average dose required in normal subjects for a positive reaction was 2·25 mg/0·1 ml, and for patients 1·04 mg/0·1 ml.

TABLE 5. Responses to skin testing with autologous tumour and normal tissue membrane extracts II: tested at ⩽0·33 mg antigen protein/0·1 ml

Disease	No. not anergic and tested at⩽0·33 mg protein/0·1 ml	No. positive reactions at⩽0·33 mg protein/ 0·1 ml (%)
Acute lymphocytic leukaemia	8	3 (38)
Chronic lymphocytic leukaemia	12	5 (42)
Lymphoma, Hodgkin's disease	3	2 (66·7)
Acute myelocytic leukaemia	13	10 (77)
Chronic myeloctytic leukaemia	9	3 (30)
Gammopathies	3	2 (66·7)
Carcinoma, melanoma, Wilm's tumour	4	2 (50)
Total	52	27 (52)
Normal volunteers	28	3 (10·7)

Effects of testing with separated control cell types and with extracts from these cells

To further define the reactions seen in normal volunteers and to rule out the possibility that the process of preparation of the extracts induced reactive materials, studies were done with separated preparations of autochthonous polymorphonuclear leucocytes, lymphocytes, and erythrocytes (Table 6). These tests, with intact cells and with extracts, were performed on some normal individuals who had previously been very reactive to autochthonous peripheral blood leucocyte membranes. One individual who gave a positive reaction to the peripheral leucocyte extract at 0·2 mg protein/0·1 ml inoculum also gave positive reactions to intact lymphocytes and granulocytes and to extracts made from these. The number of intact cells needed to elicit a positive reaction and the number of cells represented in the inoculated extracts were similar. Two other volunteers gave positive reactions to concentrations of membranes of lymphocytes and granulocytes that were similar to that of their unseparated leucocyte extracts. They did not give positive reactions to intact cells, however, when tested with up to 1×10^8 cells. The normal subjects (and a small group of cancer patients) did not respond as well to erythrocytes as to leucocytes; fewer positive reactions were seen and higher concentrations were needed to elicit them.

Results in anergic patients

Delayed reactions to autochthonous tumour extracts were seen in five patients who were unreactive to the five standard skin test antigens. Three of these patients also could not be sensitized to DCNB. The average dose of antigen protein required for a positive reaction among these five patients was high, 2·3 mg/0·1 ml; none was positive at $\leqslant$0·33 mg/0·1 ml. Biopsies were obtained of two of these reactions and the histologic pictures were not consistent with delayed hypersensitivity (polymorphonuclear leucocyte infiltration).

TABLE 6. Autologous skin tests with normal recipients

Material inoculated	No. of recipients	Positive reactions		
		Number (%)	Average No. cells/0·1 ml inoculum (range)	Average protein concentration (mg/0·1 ml) needed to elicit positive reaction (range)
Peripheral leucocytes, unseparated, membranes	29	22 (75·9)	$1{\cdot}4\times10^8$ ($0{\cdot}12$–2×10^8)	2·25 (0·15–5·0)
Lymphocytes, intact	4	1 (25)	$0{\cdot}1\times10^8$	0·38
Lymphocytes, membranes	3	2 (67)	$1{\cdot}5\times10^8$ (1–$1{\cdot}9\times10^8$)	0·98 (0·8–1·1)
Granulocytes, intact	4	1 (25)	$0{\cdot}1\times10^8$	3·0
Granulocytes, membranes	3	2 (67)	$0{\cdot}8\times10^8$ ($0{\cdot}6$–$1{\cdot}0\times10^8$)	1·1 (0·7–1·5)
Erythrocytes, intact	3	0 (0)	—	—
Erythrocytes, membranes	10	4 (10)	$2{\cdot}5\times10^9$	2·51 (0·16–5·0)

TABLE 7. Skin tests in non-anergic patients with acute lymphocytic leukaemia

Test material	Skin test results	Tests performed in:	
		Leukaemic phase	Remission
Autologous leukaemic cell membranes	Positive	5	5
	Negative	9	1
Autologous remission cell membranes	Positive	NT*	0
	Negative	NT	6

* NT = not tested.

Results in acute lymphocytic leukaemia

Although the number of patients tested with acute lymphocytic leukaemia was small, there was a tendency for non-anergic patients in relapse to be unreactive to autochthonous membranes obtained from relapse-phase leucocytes: nine of fourteen tests were negative (Table 7). Five patients tested in remission with the autochthonous leukaemic-phase leucocyte membrane preparation were positive; two of these five had converted from negative to positive and three had not been tested in relapse.

Tests were performed on five other patients with acute lymphocytic leukaemia, who were in long-term remission. Extracts of the apparently normal peripheral blood leucocytes and bone marrow of these patients gave no positive reactions at $\leqslant 0{\cdot}33$ mg protein/0·1 ml (Table 7). One patient in remission was tested at the same time with both leukaemic cell membranes and with remission cell membranes; a positive reaction was elicited only by the leukaemic extract.

Clinical correlation

Correlations were sought between presence or absence of skin reactivity in non-anergic patients and a variety of clinical parameters (Table 8). A higher percentage of positive reactors had never been treated, lived more than 12 months after the skin tests, and had more than one of the five standard skin tests positive; a lower percentage of the positive reactors had ever received radiation therapy. The number of patients involved was too small, however, for statistical significance.

TABLE 8. Correlations of reactivity to autochthonous tumour membrane extracts with clinical parameters

	Parameter			
	No. and (%) of patients evaluable for the given skin test result			
Skin test result	Survival more than 12 months	No history of any anti-tumour therapy	History of radiation therapy	More than one out of five standard skin tests+
Positive	14 (63·6)	4 (17·4)	3 (13·0)	11 (57·9)
Negative	14 (36·8)	5 (11·6)	9 (20·9)	18 (45·0)

No relationship was found between skin reactivity and total peripheral leucocyte or peripheral lymphocyte counts. 55% of both reactors and non-reactors had received prior blood, white cell or platelet transfusions. There was likewise no correlation of reactivity with a history of having received any specific anti-tumour medication (steroids, alkylating agents, vinca alkaloids, antibiotics, enzymes).

DISCUSSION

The results of the present study indicate that membrane extracts of a variety of human tumour cells are capable of eliciting a delayed skin reaction in the autochthonous host. These reactions had the typical appearance, time course, and histology of human delayed cutaneous hypersensitivity.

The specificity of the antigens producing the positive skin reactions was a major concern in this study. Contamination of the extracts with bacterial antigens (Stewart, 1969a) did not appear to be a problem in this study. Most of the tumour cells were obtained from normally sterile sites, e.g., peripheral blood, from patients without evidence of infection. Only extracts which produced no bacterial growth in culture were used for testing. The antigenicity

also did not seem to be a function of the way in which the membranes were prepared; intact cells and membranes isolated by other methods gave similar results.

As in the previous studies with tumour antigens (Hughes & Lytton, 1964; Stewart, 1969a, b; Fass *et al.*, 1970a), some positive reactions were obtained with control extracts. Quantitation of the amount of material injected, by determination of the membrane protein concentration, has aided in the interpretation of the positive reactions with control extracts. With both the patients and the normal volunteers, these reactions were elicited only by relatively high protein concentrations. In contrast, the patients had positive reactions to membrane extracts of autochthonous tumour cells even at low protein concentrations.

This observed difference in reactivity to cell extracts by the patients has several possible interpretations: (a) tumour patients could have increased reactivity to normal cell antigens and thus react with such components at lower concentrations; (b) the tumour extracts might have a higher concentration of normal cell antigens; or (c) the reactions seen to control extracts at high protein concentration and the reactions to tumour extracts at low concentrations might be qualitatively different, the latter representing at least in part, reactions to tumour-specific antigens.

There is not much evidence to support the concept of heightened reactivity of the tumour patients to normal cell components. The observed reactivity of the cancer patients against their tumour occurred at a time when their ability to mount a cellular response to a battery of standard antigens was less than that of the normal subjects. In four of the non-leukaemic patients, tests with extracts of normal cells were positive only at high protein concentrations. In the studies on patients with leukaemia, it was not possible in most instances to obtain suitable control cells along with the tumour cells. However, patients with acute lymphocytic leukaemia in remission gave negative reactions to extracts of autochthonous remission cells.

Higher concentration of normal cell antigens in the tumour extracts is a possible explanation for some of the observed reactions. However, assays for HL-A histocompatibility antigens on the extracts (inhibition of cytotoxic activity of anti-HL-A alloantisera), done with the cooperation of Dr Dean Mann, have shown that the control extracts generally contained at least as much HL-A antigen/mg protein as the tumour extracts.

The normal volunteers in this study reacted to autochthonous leucocyte membranes in relatively high doses. The nature of these reactions is not completely clear. They could be due to non-specific inflammation induced by the large amount of protein. Alternatively, 'non-specific' reactions could have been produced by the presence of mediators of delayed hypersensitivity reactions in the extracts. However, migration inhibitory factor (David, 1966; Bloom & Bennett, 1968) and leucotactic factor (Ward, Remold & David, 1969) have been shown to be freely soluble proteins. Reactions were obtained here with the particulate membrane extract and not with the 105,000 *g* supernatant fraction. In addition, lymphocytes have been shown to be the principal cells which elaborate the mediator substances (Ward *et al.*, 1969). Positive skin reactions were obtained here with extracts of polymorphonuclear leucocytes, and occasionally even with erythrocyte extracts.

The reactions produced in normal individuals may have been true delayed hypersensitivity reactions to antigenic determinants on the autochthonous leucocyte membrane. Serum autoantibodies have been found in normal animals (Boss, Silber & Nelken, 1968; Weir & Elson, 1969) and in man (Herberman, 1969; McDuffie, 1970). It is possible that both humoral and cellular autoimmune reactivity are present in the normal state, and could even play important functional roles (Grabar, 1958; Boyden, 1964).

Until more is known about the nature of the reactivity to normal leucocytes, it cannot be definitely concluded that the reactions of patients to tumour extracts are tumour specific. It may be that we are detecting true cellular autoimmunity in controls and a summation of this and of immunity to tumour-specific antigens in patients.

Some available evidence indicates that skin reactivity to autochthonous tumour membranes in patients may be related to their clinical state. Burkitt's lymphoma and malignant melanoma are two human cancers for which evidence exists for effective host immune defence mechanisms (see Fass *et al.*, 1970a, b). In both of these diseases, reactivity to autochthonous tumour membrane extracts has been shown to correlate with the clinical status of the patients (Fass *et al.*, 1970a, b). Bernard *et al.* (1954) and Skurkovich *et al.* (1968) were able to correlate the presence of intradermal reactivity to extracts of leukaemic leucocytes in subjects with acute leukaemia with a state of remission. Our results with patients with acute lymphocytic leukaemia appear to confirm these observations. In addition, in our patient series as a whole, reactive patients tended to survive longer after the skin tests.

Extensive studies are presently in progress in an attempt to identify the components of the cell membrane responsible for normal and for tumour reactivity. A variety of solubilization and separation procedures are being performed. This approach has already been successful in studies of patients with carcinoma of the lower digestive tract (Hollinshead *et al.*, 1970). Soluble fractions obtained from the membranes of autochthonous carcinomas of the colon and rectum were shown to be capable of producing delayed hypersensitivity reactions. Carcinoembryonic antigen was found in the reactive fractions. Similar studies are underway with the membranes of lymphomas, leukaemias and other malignancies.

ACKNOWLEDGMENT

We acknowledge with thanks the excellent technical assistance of Miss Myrthel Nunn.

REFERENCES

BERNARD, J., GRABAR, P., SELIGMANN, M. & BADILLET, M. (1954) Intradermoréactions á des extraits de leucocytes normaux et de leucocytes leucémiques chez des sujets atteints de leucémie aiguë. *Bull. Mem. Soc. Med. Hôp. (Paris)*, **70**, 1169.

BLOOM, B.R. & BENNETT, B. (1968) Migration inhibitory factor associated with delayed type hypersensitivity. *Fed. Proc.* **27**, 13.

BOONE, C.W., FORD, L.E., BOND, H.E., STUART, D.C. & LORENZ, D. (1969) Isolation of membrane fragments from HeLa cells. *J. Cell Biol.* **41**, 378.

BOSS, J.H., SILBER, E. & NELKEN, D. (1968) Patterns of naturally occurring circulating antibodies to rat tissue antigens in the rat. *Path. Microbiol.* **31**, 1.

BOYDEN, S. (1964) Autoimmunity and inflammation. *Nature (Lond.)*, **201**, 200.

BRENT, L., BROWN, J. & MEDAWAR, P.B. (1958) Skin transplantation immunity in relation to hypersensitivity. *Lancet*, **ii**, 561.

BROWN, R.S., HAYNES, H.A., FOLEY, H.T., GODWIN, H.A., BERARD, C.W. & CARBONE, P.P. (1967) Hodgkin's disease: Immunologic, clinical and histologic features of 50 untreated patients. *Ann. intern. Med.* **67**, 291.

CHURCHILL, W.H., JR, RAPP, H.J., KRONMAN, B.S. & BORSOS, T. (1968) Detection of antigens of a new diethylnitrosamine-induced transplantable hepatoma by delayed hypersensitivity. *J. nat. Cancer Inst.* **41**, 13.

DAVID, J.R. (1966) Delayed hypersensitivity in vitro: Its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc. nat. Acad. Sci. (Wash.)*, **56**, 72.

DAVIES, D.A.L. (1966) Mouse histocompatibility antigens derived from normal and from tumour cells. *Immunology*, **11**, 115.

FASS, L., HERBERMAN, R.B. & ZIEGLER, J.L. (1970a) Delayed cutaneous hypersensitivity reactions to autologous extracts of Burkitt's lymphoma cells. *New Engl. J. Med.* **282**, 776.

FASS, L., HERBERMAN, R.B., ZIEGLER, J.L. & KIRYABWIRE, J.W.M. (1970b) Cutaneous hypersensitivity reactions to autologous extracts of malignant melanoma cells. *Lancet*, **i**, 116.

GRABAR, P. (1958) Some theoretical aspects of the problem of autoantibodies. In *Proc. Sixth Int. Cong. Haematol.* p. 832. Grune and Stratton, New York.

GREENWALT, T.J., GAJEWSKI, M. & MCKENNA, J.L. (1962) A new method for preparing buffy coat-poor blood. *Transfusion*, **2**, 221.

HELLSTRÖM, K.E. & MÖLLER, G. (1965) Immunological and immunogenetic aspects of tumour transplantation. *Progr. Allergy*, **9**, 158.

HERBERMAN, R.B. (1969) Studies on the specificity of human cytotoxic antibody reactive with cultures of lymphoid cells. *J. nat. Cancer Inst.* **42**, 69.

HERBERMAN, R.B. & OREN, M.E. (1969) Delayed cutaneous hypersensitivity reactions to membrane extracts of human tumor cells. *Clin. Res.* **17**, 403.

HOLLINSHEAD, A., GLEW, D., BUNNAG, B., GOLD, P. & HERBERMAN, R.B. (1970) Study of skin reactive soluble antigen from intestinal cancer cell membranes and relationships to carcinoembryonic antigens. *Lancet*, **i**, 1191.

HUGHES, L.E. & LYTTON, B. (1964) Antigenic properties of human tumors: Delayed cutaneous hypersensitivity reactions. *Brit. med. J.* **i**, 209.

KAHAN, B.D. (1967) Cutaneous hypersensitivity reactions of guinea pigs to proteinaceous transplantation antigen. *J. Immunol.* **99**, 1121.

LOWRY, O.H., ROSEBROUGH, N.J. & FARR, A.L. (1952) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MANN, D.L., ROGENTINE, G.N., FAHEY, J.L. & NATHENSON, S.G. (1968) Solubilization of human leucocyte membrane isoantigens. *Nature (Lond.)*, **217**, 1180.

MCCOLLESTER, D.L. & SEMENTE, G. (1966) Membrane isolation and cytoskeletal breakdown. II. Enzyme studies revealing cytoskeletal stabilization by FAD. *Exp. Cell Res.* **42**, 209.

MCDUFFIE, F.C. (1970) Autoantibodies in healthy subjects. *Ann. intern. Med.* **72**, 596.

OREN, M.E. & HERBERMAN, R.B. (1970) Delayed hypersensitivity reactions to human tumour membrane extracts. *Proc. Xth Int. Cancer Cong.*, Houston, Texas, U.S.A., p. 223.

SKURKOVICH, S.V., RIZNICHENKO, F.M., KAVERZNEVA, M.M., MAKHONOVA, L.A. & CHERVONSKII, G.I. (1968) Reaction of delayed hypersensitivity in children suffering from acute leukaemia (in Russian). *Probl. Gemat.* **13**, 33.

STEWART, T.H.M. (1969a) The presence of delayed hypersensitivity reactions in patients toward cellular extracts of their malignant tumors. I. The role of tissue antigen, nonspecific reactions of nuclear material and bacterial antigen as a cause for this phenomenon. *Cancer*, **23**, 1368.

STEWART, T.H.M. (1969b) The presence of delayed hypersensitivity reactions in patients toward cellular extracts of their malignant tumors. II. A correlation between the histologic picture of lymphocyte infiltration of the tumor stroma, the presence of such a reaction, and a discussion of the significance of this phenomenon. *Cancer*, **23**, 1380.

WARD, P.A., REMOLD, H.G. & DAVID, J.R. (1969) Leukotactic factor produced by sensitized lymphocytes. *Science*, **163**, 1079.

WARREN, L., GLICK, M.C. & NASS, M.K. (1967) Membranes of animal cells. I. Methods of isolation of the surface membrane. *J. cell. Physiol.* **68**, 269.

WEIR, D.M. & ELSON, C.J. (1969) Antitissue antibodies and immunolgical tolerance to self. *Arthr. Rheum.* **12**, 254.

ABBREVIATION

AML acute myelocytic leukaemia