Biosynthesis of the First Component of Complement by Human Fibroblasts

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1. Haemolytic activity corresponding to that of the first component of complement (C1) was synthesized and secreted by all nine human fibroblast cell lines examined. No activity was found in the culture media of a variety of other human cell lines. 2. The component-C1 haemolytic activity secreted by the fibroblast lines behaved in an identical manner, in most respects, with that of the component-C1 haemolytic activity of human serum. The component-C1 haemolytic activity secreted by fibroblasts, however, was less susceptible to inhibition by rabbit fragment F(ab')₂ anti-(human subcomponent C1q) than was the component-C1 haemolytic activity of human serum. 3. Biosynthesis of fibroblast component-C1 haemolytic activity was inhibited by the presence of cycloheximide and regained on its removal. 4. Incorporation of radioactivity into proteins secreted by the fibroblasts and release of component-C1 haemolytic activity by the fibroblasts both increased in a linear manner until several days after the cultures had reached a state of confluent growth. 5. Radioactivity was incorporated into sub-components C1q, C1r and C1s, as judged by the formation of specific immunoprecipitates and by absorption with immune aggregates. 6. The immunoprecipitates formed by using antisera against subcomponents C1r and C1s were run on polyacrylamide gels in sodium dodecyl sulphate, and this provided convincing physicochemical evidence for the biosynthesis of these subcomponents *de novo*. 7. The results obtained with immunoprecipitates formed by using anti-(subcomponent C1q) suggest that subcomponent C1q may be synthesized and secreted by fibroblast cell lines *in vitro*, in a form with a higher molecular weight than that of subcomponent C1q which is isolated by conventional techniques of protein fractionation from fresh serum.

The first component of complement (C1) is a Ca²⁺-dependent complex composed of the three subcomponents C1q, C1r and C1s (Lepow *et al.*, 1963; Gigli *et al.*, 1976). All three subcomponents have been reported to be synthesized, as estimated by the formation of component-C1 haemolytic activity, by the columnar cells of the small and large intestine in the guinea pig (Colten *et al.*, 1966, 1968). Other studies, using immunochemical techniques, have suggested that human and monkey subcomponent C1q may be produced by a variety of tissues such as the liver, spleen, bone marrow, lung and macrophages (Stecher *et al.*, 1967). In another study, Day *et al.* (1970) presented evidence that, in the pig, non-intestinal tissues, particularly those rich in lymphoid cells, produced material antigenically similar to subcomponent C1q. Bing *et al.* (1975) have shown that long-term primary suspension cultures of normal human colon, adenocarcinoma of the colon and transitional epithelial cells of the urogenital tract synthesized the entire component-C1 complex, as judged by haemolytic activity and, in certain cases, by immunochemical techniques. These latter

results are considered to be consistent with the original findings by Colten *et al.* (1966, 1968) (see Colten, 1976, for review), since it is believed that epithelial cells of the urogenital tract and colon may be of common origin.

Al-Adnani & McGee (1976) have shown, by immunoperoxidase and immunoprecipitation procedures, that material antigenically similar to subcomponent C1q is synthesized and secreted by human and rat fibroblast cell lines. However, Bing *et al.* (1975) found no evidence for synthesis of the component-C1 complex in the one fibroblast line that they examined. In the present paper we report the biosynthesis of component-C1 haemolytic activity by nine fibroblast cell lines and the incorporation of radioactivity into material that behaves immunochemically and physicochemically in a similar fashion to subcomponents C1r and C1s. Evidence was obtained that subcomponent C1q may be synthesized by fibroblasts in culture and secreted in a form with an apparently higher molecular weight than that of subcomponent C1q isolated from normal human serum.

Materials and Methods

Cell lines used

Nine human fibroblast cultures were tested for the ability to synthesize component-C1 haemolytic activity. Of these, eight were primary explants from skin biopsies or lung tissue. Three were from foetal tissue and five from adult tissue. The ages of the cells, as shown in Table 1, are presented as transfer generations, i.e. the number of times the cells were transferred serially (usually at a ratio of 1:4). LNSV-40 (Table 1) is an SV-40-virus-transformed fibroblast cell line from a patient with the Lesch-Nyhan syndrome. Other human cell lines tested are shown in Table 1. The following people have kindly supplied some of the cell lines: Dr. B. Noël (DUV), Laboratoire de Cytogénétique, Chambéry, France; Dr. M. Bobrow (EIJ061), Genetics Laboratory, University of Oxford; Dr. J. Watkins (HT55), Department of Medical Microbiology, Welsh National School of Medicine, Cardiff, Wales, U.K.

Culture conditions used to grow cells

Cells were grown at 37°C for several generations in RPMI/1640 medium (Gibco Bio-Cult, Paisley, Renfrewshire, Scotland, U.K.) with 10% (v/v) foetal calf serum plus penicillin (100 units/ml) and streptomycin (100 µg/ml). For measurement of component-C1 haemolytic activity, and for the radioisotope-labelling experiments, the cells were transferred to the same medium with 10% (v/v) heat-

inactivated foetal calf serum. Foetal calf serum was obtained from Gibco Bio-Cult and was inactivated by heating at 56°C for 30 min, which destroys the bovine component-C1 haemolytic activity. Three batches of foetal calf serum were tested. In one of these, all the fibroblasts tested gave approx. 10–15-fold higher amounts of component-C1 haemolytic activity. Fibroblasts grown in RPMI1640 medium made 20% (v/v) with respect to inactivated human AB serum (Blood Transfusion Centre, Oxford, U.K.) gave low activity.

Lymphocytes from peripheral blood were separated on a Ficoll/Triosil gradient (Bøyum, 1968) and cultured in RPMI/1640 medium made 20% (v/v) with inactivated human AB serum and 1% (w/v) with phytohaemagglutinin.

Fibroblasts that were growing well had a doubling time of about 24 h, i.e. they reached confluency at days 3 and 4 of the experiment and gave the very tightly packed monolayer typical of fibroblasts in culture. Fibroblasts that appeared to be reaching senescence had longer doubling times and never appeared to be very tightly packed. These cells appeared to have stopped producing detectable amounts of component-C1 haemolytic activity.

The number of cells in the fibroblast cultures was approx. 2×10^6 when confluent, and about 5×10^6 for the D98/AH2 cell line. The lymphocyte culture was from 5 ml of blood and contained about 6×10^6 lymphocytes in 60 ml of medium. The lymphoid lines were grown in suspension to about

Table 1. *Cell lines examined for the ability to synthesize and secrete component-C1 haemolytic activity*

Full experimental details of the culture conditions used to grow the cells and a description of the assay of component-C1 haemolytic activity are given in the text. All the fibroblast lines tested released component-C1 haemolytic activity into the culture medium, whereas the media of all the non-fibroblast lines showed no detectable component-C1 haemolytic activity in the assay used. Most cell lines not given a reference were developed in the Genetics Laboratory, University of Oxford, Oxford, U.K., where they are in routine use.

Cell type	Source	Transfer generation	Mode of growth	Reference
Fibroblast RW	♂ Adult skin (primary)	30–35	Attached	Solomon <i>et al.</i> (1976)
Fibroblast BREN	♀ Adult skin (primary)	11–12	Attached	
Fibroblast DUV	♀ Adult skin (primary)	5–11	Attached	
Fibroblast EIJO61	♀ Adult skin (primary)	9	Attached	
Fibroblast IMR90	♀ Foetal lung (primary)	29	Attached	Nichols <i>et al.</i> (1977)
Fibroblast TO2	♂ Foetal skin (primary)	5	Attached	
Fibroblast WI38	Foetal lung (primary)		Attached	Croce <i>et al.</i> (1973)
Fibroblast LNSV-40	♂ Adult skin (SV-40-transformed)		Attached	
Fibroblast HM	♂ Adult skin (primary)	12	Attached	
Peripheral blood lymphocytes	♂ Normal adult		Suspension	Klein <i>et al.</i> (1967)
DAUDI	♂ B-cell lymphoid line (Burkitt's lymphoma)		Suspension	
Bristol 8	B-cell lymphoid line		Suspension	Goodfellow <i>et al.</i> (1976)
MOLT4	T-cell lymphoid line		Suspension	Minowada <i>et al.</i> (1972)
D98/AH2 (HeLa)	♀ Cervical carcinoma		Attached	Szybalski <i>et al.</i> (1962)
HT55	Adenocarcinoma (rectum)		Attached	Watkins & Sanger (1977)
IMR32	♂ Neuroblastoma		Attached	Tumilowicz <i>et al.</i> (1970)

5×10^5 – 1×10^6 cells/ml. The number of HT55 and IMR90 cells could not be accurately determined because the cells grew in clumps, but they were estimated to be approximately the same number as found in the fibroblast cultures.

Radioactive labelling

For long-term (24–72 h) labelling of the subcomponents of C1, radioactive amino acids were added directly to the culture medium containing heat-inactivated foetal calf serum. In short-term (1–6 h) labelling studies the radioactive amino acids were added to culture medium that was free of both foetal calf serum and the amino acids being added. Labelled compounds were all obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The amino acids and amounts used per bottle of 2×10^6 cells were: L-[^{35}S]methionine (50 μCi ; 339 mCi/mmol); [2- ^3H]glycine (50 μCi ; 5.3 mCi/mmol); L-phenyl[2,3- ^3H]alanine (50 μCi ; 16.6 mCi/mmol); L-[6- ^3H]proline (50 μCi ; 677 mCi/mmol) or L-[3,4(n)- ^3H]proline (50 μCi ; 40 Ci/mmol).

Inhibition of biosynthesis of component-C1 haemolytic activity by cycloheximide

Two identical cultures of DUV fibroblasts, grown in parallel, were monitored for component-C1 haemolytic activity. On day 6 the medium was removed and medium containing cycloheximide (200 $\mu\text{g}/\text{ml}$) was added to each culture. On day 8 the cycloheximide was removed from one bottle by removing the medium and washing the monolayer with fresh medium. Fresh medium was then added back to this bottle and monitoring of the component-C1 haemolytic activity released into the culture media of both cultures was continued (Fig. 5).

Assay of component-C1 haemolytic activity in culture media

Preparation of the reagents and buffers used in the haemolytic assays was performed as described by Reid *et al.* (1977). Dilutions of the cell culture medium (0.5 ml), made in veronal-buffered saline assay medium, were incubated with EAC4 cells (0.5 ml, 1×10^8 cells/ml) at 30°C for 20 min, then ice-cold veronal-buffered saline assay medium (1.0 ml) was added and the cells were spun down at 1000g for 10 min. The cell pellet was resuspended in assay buffer (1.0 ml) and incubated at 30°C for 60 min, then component C2 (0.5 ml, 200 effective molecules/ml) was added and the mixture incubated at 30°C for 15 min. EDTA buffer (0.5 ml), pH 7.4, was then added, and finally the reagent C-EDTA (1.0 ml) was added and incubation continued for 1 h at 37°C. The degree of lysis was determined by reading the $A_{412}^{1\text{cm}}$ of the 1000g supernatant after addition of 4.5 ml of 0.15 M-NaCl.

Antisera to subcomponents C1q, C1r and C1s

Rabbit anti-(human subcomponent C1q) was prepared as described by Reid *et al.* (1972). Antisera to human subcomponents C1r and C1s were provided by Dr. R. B. Sim and were prepared as described by Sim *et al.* (1977).

IgG* and fragment F(ab')₂ were prepared from normal rabbit serum and rabbit anti-(human subcomponent C1q) serum as described by Reid (1971).

Liquid-scintillation counting of radioactivity in suspensions and in polyacrylamide-gel slices

Suspension (10–200 μl) or gel slice (1.2–1.5 mm) was placed in liquid-scintillation vials and 1.0 ml of a solution of water/NCS tissue solubilizer (1:9, v/v) added. Vials were incubated at 50°C for 2 h; then, after cooling, 10 ml of toluene containing 0.5% (w/v) PPO (2,5-diphenyloxazole) and 0.03% (w/v) dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] was added to each vial. Samples were counted for radioactivity in an LKB-Wallac 1210 Ultrabeta counter.

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate

Polyacrylamide-gel electrophoresis in SDS was performed as described by Fairbanks *et al.* (1971).

Preparation of human subcomponent C1q

Human subcomponent C1q was prepared as described by Reid (1974).

Immunoprecipitation, or absorption with immune aggregates, of the C1 subcomponents from radioactively labelled fibroblast culture media

(1) *Immunoprecipitation of subcomponent C1q.* A solution of carrier subcomponent C1q (0.3–0.6 ml, 1000 $\mu\text{g}/\text{ml}$) was added to the culture medium (20–30 ml) obtained from radioactive-labelling experiments. The mixture was made 10 mM with respect to EDTA, then it was concentrated to approx. 1.8 ml in an Amicon Diaflo 10 ml cell by using a PM-10 membrane. The concentrate was mixed with enough heat-inactivated rabbit anti-(human subcomponent C1q) to precipitate all the unlabelled carrier subcomponent C1q added. [The rabbit anti-(human subcomponent C1q) had been dialysed extensively against 10 mM-EDTA/150 mM-NaCl, pH 7.4, before use.] The mixture of concentrate plus antiserum was incubated at 37°C for 2 h, then at 4°C for 16 h. The immunoprecipitate formed was washed with 5×0.5 ml of ice-cold 10 mM-EDTA/150 mM-NaCl, pH 7.4. Samples of this subcomponent-C1q immunoprecipitate were then taken for measurement of radioactivity or for electrophoresis on polyacrylamide gels in the presence of SDS.

* Abbreviations: IgG, immunoglobulin G; SDS, sodium dodecyl sulphate.

(2) *Immunoprecipitation of the proenzyme forms of subcomponents C1r and C1s.* To the first supernatant obtained after centrifuging down (2000g, 15 min) the subcomponent-C1q immunoprecipitate, human serum (1.0 ml) was added (the human serum had been dialysed against 10 mM-EDTA/150 mM-NaCl, pH 7.4, and contained approx. 60 μ g of each of the proenzyme forms of subcomponents C1r and C1s). Then enough rabbit anti-(human subcomponent C1r) and rabbit anti-(human subcomponent C1s) was added to precipitate all the unlabelled carrier proenzyme forms of subcomponents C1r and C1s over 16 h at 4°C. The subcomponent C1q in the human serum was precipitated at this step also by virtue of its binding affinity for immune aggregates. The immunoprecipitate was allowed to form and was washed in exactly the same manner as for the subcomponent-C1q immunoprecipitate.

(3) *Immunoprecipitation of the activated forms of subcomponents C1r and C1s.* Concentrated radioactively labelled fibroblast-culture medium (1.8 ml) was incubated with ovalbumin-anti-ovalbumin immune aggregates (0.15 ml, 1.77 mg of antibody-antigen aggregate/ml; the ratio, by wt., of antibody to antigen was 9.4:1.0) at 37°C for 2 h and then made 10 mM with respect to EDTA. The immune aggregates were removed by centrifugation (2000g, 15 min). To the supernatant, human serum (1.0 ml), or a partially purified activated C1 preparation (1.0 ml, containing 50 μ g each of the activated forms of subcomponents C1r and C1s), was added. The immunoprecipitation and washing procedures then used were identical with those described above, except that all solutions were made 5 mM with respect to CaCl_2 and a 2 h incubation at 37°C was included before the immunoprecipitation step for 16 h at 4°C.

(4) *Binding of subcomponent C1q to immune aggregates.* A solution of carrier subcomponent C1q (0.2 ml, 1000 μ g/ml) was added to radioactively labelled fibroblast culture medium (20–30 ml). The mixture was made 10 mM with respect to EDTA and then concentrated to 1.8 ml in an Amicon Diaflo 10 ml cell with a PM-10 membrane. Immune aggregates composed of ovalbumin and anti-ovalbumin (0.3 ml, 1.77 mg of antibody aggregate/ml) were added to the concentrated labelled medium and incubated at 30°C for 30 min and then at 4°C for 16 h. The suspension was centrifuged (2000g, 15 min) and the precipitate washed with 5 $\times$ 0.5 ml of ice-cold 10 mM-EDTA/150 mM-NaCl, pH 7.4. Samples of this precipitate were taken for radioactivity counting and SDS/polyacrylamide-gel electrophoresis.

Binding of radioactively labelled material, from fibroblast culture medium, to antibody-sensitized erythrocytes

Radioactively labelled material from fibroblast culture medium (20 ml) was concentrated to 1.6 ml.

Samples of the concentrate (0.15 ml) were added to antibody-sensitized cells (0.5 ml, 2×10^8 cells/ml) or unsensitized cells (0.5 ml, 2×10^8 cells/ml). The mixture was incubated at 30°C for 30 min, then spun down (1000g, 10 min). The supernatant was kept for assay of component-C1 haemolytic activity, and the pellet was resuspended in assay buffer (0.75 ml) and then extensively washed (with 7 $\times$ 0.75 ml) with assay buffer. After the final wash the packed cells were dissolved in NCS tissue solubilizer (1.0 ml), and the amount of bound radioactivity was determined.

Inhibition of fibroblast and human serum component-C1 haemolytic activity by rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q)

Dilutions (0.5 ml, 8×10^8 effective component-C1 molecules/ml) of fibroblast medium, from a culture that had been growing for 9 days, were incubated at 30°C for 14 min with increasing dilutions (1:10 to 1:20480) of rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q) (0.5 ml, stock solution 1.21 mg/ml) or normal rabbit fragment $F(ab')_2$ (0.5 ml, stock solution 2.50 mg/ml). Dilutions (0.5 ml, 3.6×10^9 effective component-C1 molecules/ml) of normal human serum in place of fibroblast medium were treated in the same manner. EAC4 cells (0.5 ml, 1×10^8 cells/ml) were then added and the assay for component-C1 haemolytic activity was carried out as described above.

Binding of fibroblast and human serum component-C1 haemolytic activity by preformed immune aggregates

Dilutions of fibroblast medium (0.5 ml, 2.9×10^8 effective component-C1 molecules/ml) or human serum (0.5 ml, 1.6×10^8 effective component-C1 molecules/ml) were incubated at 30°C for 15 min with increasing dilutions (1:5–1:20480) of an antibody-antigen suspension (0.5 ml, stock solution 4.65 mg of antibody aggregate/ml). EAC4 cells (0.5 ml, 1×10^8 cells/ml) were then added, and the assay for component-C1 haemolytic activity was carried out as described above.

Results

Cell lines examined for the ability to synthesize and secrete component-C1 haemolytic activity into the culture medium

All nine human fibroblast cell lines listed in Table 1 synthesized and released component-C1 haemolytic activity to approximately the same amount when grown in medium containing the same batch of heat-inactivated foetal calf serum. As shown in Fig. 1, the fibroblast component-C1 haemolytic activity was released into the culture fluid in a linear manner with time, until at least 3–4 days after the cells had reached confluent growth. The amount of component-C1 haemolytic activity present in the culture media of

these fibroblast cell lines was normally in the range 1×10^9 – 4×10^9 'effective' molecules/ml 2–3 days after the stage of confluent growth had been reached. Since in most fibroblast cultures there was 20 ml of culture medium and approx. 2×10^6 cells at confluence, it can be estimated that each fibroblast cell synthesized and secreted approx. 1×10^4 – 4×10^4 'effective' molecules of component C1 in 7–8 days of culture. A typical titration performed on 0.5 ml of unconcentrated fibroblast medium to determine the amount of component-C1 haemolytic activity is shown in Fig. 2. By using the same assay procedure values of 16×10^{12} – 20×10^{12} effective molecules of component C1/ml of normal human serum were

Fig. 1. Release of component-C1 haemolytic activity and trichloroacetic acid-precipitable protein into the culture medium by human fibroblasts

BREN fibroblasts were cultured in the presence of L-[¹⁴C]proline. Full experimental details are given in the text. Samples of the culture medium were examined for the presence of component-C1 haemolytic activity and for the presence of radioactivity in trichloroacetic acid-precipitable proteins at 0, 2, 5, 7 and 9 days. The culture had reached confluence after 5–6 days. O, Percentage lysis of 1.5×10^8 sensitized erythrocytes (used in the assay of component-C1 haemolytic activity) produced by a 1:20 dilution of the culture medium; ▲, radioactivity (c.p.m.) present in the trichloroacetic acid-precipitable protein derived from 0.4 ml of culture medium. Full details of the assay used for the determination of component-C1 haemolytic activity are given in the text.

obtained. The value obtained in Fig. 2 for the 7-day DUV culture medium was 2.3×10^9 effective molecules/ml. The starting culture medium and the culture media from other, non-fibroblast, cell lines (Table 1) all gave negative values when tested for component-C1 haemolytic activity. This showed that, if these cells did synthesize the component-C1 complex, then they did not release it into the culture medium, or, if it was released, it was present at an amount at which it could not be detected by the haemolytic assay used. The number of cells per ml in the culture media of these non-fibroblast cell lines was approximately the same as, or greater than, that obtained in the fibroblast cultures.

In the radioactive-labelling studies only three of the fibroblast cell lines were used, the DUV, HM and LNSV-40 lines, which were all derived from adult skin. LNSV-40 fibroblasts are an SV-40-virus-transformed line. In view of the apparent dependence of the magnitude of the release of component-C1 haemolytic activity, by the fibroblasts, on the batch of foetal calf serum used, and perhaps other experimental factors, the cultures were routinely checked for adequate synthesis of component-C1 haemolytic activity after 2–3 days' growth, before they were used for radioactive-labelling experiments. Recent experiments have indicated that variability in the amounts of component-C1 haemolytic activity found in fibroblast culture medium is primarily due to either lack of subcomponent-C1q synthesis and secretion or inactivation of subcomponent C1q. In these experiments addition of purified human subcomponent C1q (0.5 ml, 50–200 ng/ml) to fibroblast culture medium (0.5 ml dilutions) showing very low component-C1 haemolytic activity (less than 1×10^8 effective molecules/ml) resulted in the generation of component-C1 haemolytic activity of the order of 1×10^9 – 5×10^9 effective molecules/ml.

Similarities between the component-C1 haemolytic activity released by human fibroblasts and the component-C1 haemolytic activity found in human serum

The presence of a component-C1 haemolytic activity in the culture media of human fibroblasts which appears identical with that found in human serum was shown in four ways.

(1) Specificity in the assay for component-C1 haemolytic activity. In the lysis of sheep erythrocytes there was an absolute requirement for antibody to sheep erythrocytes, components C4, C2 and components C3–C9 in addition to the activity from the fibroblast culture medium (Table 2). The component C1 haemolytic activity found in the fibroblast culture medium also displayed a specific binding role, since it was efficiently bound on to erythrocytes sensitized with antibody, but not to unsensitized erythrocytes (Table 2).

(2) Inactivation by heat and by treatment with di-

Fig. 2. Titration of the component-C1 haemolytic activity released into fibroblast culture medium

Unconcentrated fibroblast culture medium (0.5 ml) from a 7-day culture of DUV fibroblasts was used in a titration of the number of effective, or functionally active, component-C1 molecules. y is the percentage lysis of the erythrocytes used in the assay, and $-\ln(1-y)$ is plotted against 2-fold dilutions of the culture medium. The dilution at which $-\ln(1-y)=1$ is that at which an average of one haemolytically effective molecule of component C1 is offered per erythrocyte used in the assay (0.5 ml, 1×10^8 cells/ml). No component-C1 haemolytic activity was found in culture medium that was incubated without fibroblasts. Full details of the assay used for the determination of component-C1 haemolytic activity are given in the text.

Table 2. Demonstration of the specificity of the component-C1 haemolytic activity released into the culture medium by human fibroblasts

Abbreviations: F, 1:15 (v/v) dilution of DUV fibroblast culture medium (0.5 ml) from a 6-day culture; E, sheep erythrocytes (0.5 ml, 1×10^8 cells/ml); EA, sheep erythrocytes (0.5 ml, 1×10^8 cells/ml) sensitized with rabbit antibody; C2, C4, second and fourth components of complement; C-EDTA, source of components C3-C9; EAC4, sheep erythrocytes sensitized with antibody, which also have component C4 bound on to them. Full experimental details of the assay procedure are given in the text.

Reagents used in assay	Lysis of erythrocytes (%)
E+F+C4+C2+C-EDTA	4
EA+F+C4+C2+C-EDTA	80
EA+F+C2+C-EDTA	4
EA+F+C4+C-EDTA	5
EAC4+F+C2+C-EDTA	95
EAC4+C2+C-EDTA	5
EAC4+starting medium+C2+C-EDTA	8
EAC4+F(heated to 56°C)+C2+C-EDTA	4

isopropyl phosphorofluoridate. It was found that, similarly to the component-C1 haemolytic activity found in serum, the component-C1 haemolytic activity secreted by fibroblasts was completely lost by heating at 56°C for 30 min (Table 2) and also was inhibited by 2 mM-di-isopropyl phosphorofluoridate after it had been activated by complexing with cell-bound antibody.

(3) Fixation by immune aggregates. Both fibroblast component-C1 haemolytic activity and normal human serum component-C1 haemolytic activity were readily fixed by preformed immune aggregates made from rabbit anti-ovalbumin and ovalbumin (Fig. 3). The degree of fixation of samples of fibroblast, and human serum component-C1 haemolytic activity, which had approximately equivalent C1 haemolytic titres, was almost identical (Fig. 3). This suggests that both fibroblast and human serum component-C1 haemolytic activity are bound to immune complexes by the same mechanism.

(4) Inactivation by rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q). Both fibroblast and human serum component-C1 haemolytic activity

Fig. 3. Inhibition of fibroblast and human serum component-C1 haemolytic activity by immune aggregates

Samples (0.5ml) of fibroblast culture medium (2.9×10^8 effective component-C1 molecules/ml) and samples (0.5ml) of human serum (1.6×10^8 effective component-C1 molecules/ml) were incubated with increasing dilutions of preformed rabbit anti-ovalbumin immune aggregates and then assayed for the presence of component-C1 haemolytic activity. Full experimental details are given in the text. ○, Percentage inhibition of lysis of the sensitized erythrocytes used in the component-C1 haemolytic assay produced by the addition of immune aggregates to fibroblast culture medium; ▲, percentage inhibition of lysis of sensitized erythrocytes used in the component-C1 haemolytic assay produced by the addition of immune aggregates to human serum.

were inactivated by rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q) (Fig. 4) under conditions where no inactivation by equivalent concentrations of normal rabbit fragment $F(ab')_2$ took place (Fig. 4). However, the component-C1 haemolytic activity from normal human serum was far more readily inactivated than was the fibroblast component-C1 haemolytic activity by the rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q) (Fig. 4) even when over four times the amount of component-C1 activity found in human serum, relative to the component-C1 activity secreted by fibroblasts, was used.

Evidence for synthesis *de novo* of component-C1 haemolytic activity by fibroblasts

As shown in Fig. 1 fibroblast component-C1 haemolytic activity was released into the culture medium, in a linear manner, until at least 3–4 days after the cells had reached confluent growth. When fibroblasts were taken after 6 days of culture and were washed and resuspended in fresh culture medium, containing cycloheximide, only very low component-C1 haemolytic activity could be detected (Fig. 5). However, after removal of the culture medium containing cycloheximide and replacing it with fresh culture medium, the rate of release of component-C1 haemolytic activity from the fibroblasts approached that observed during the first 6 days of culture (Fig. 5).

Evidence that protein biosynthesis *de novo* was taking place was provided by the incorporation of radioactivity into trichloroacetic acid-precipitable protein, in a linear manner, for up to 3–4 days after the cells had reached confluence (Fig. 1).

Incorporation of radioactivity into proteins released from fibroblasts that have similar functional and physicochemical characteristics to the subcomponents of component C1

(1) Binding of radioactivity from fibroblast culture medium to erythrocytes coated with antibody. The amount of radioactivity bound to antibody-sensitized erythrocytes when they were incubated with radioactively labelled fibroblast medium, under the conditions described in the Materials and Methods section, was 1104 (S.D. ± 96) c.p.m. (average of five samples). The amount of radioactivity bound to unsensitized erythrocytes was 723 (S.D. ± 102) c.p.m. (average of five samples). The conditions used to perform the binding removed over 90% of the

Fig. 4. Inhibition of fibroblast and human serum component-C1 haemolytic activity by rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q)

Samples (0.5ml) of fibroblast culture medium (8×10^8 effective component-C1 molecules/ml) and samples (0.5ml) of human serum (3.6×10^9 effective component-C1 molecules/ml) were incubated with increasing dilutions of rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q) or normal rabbit fragment $F(ab')_2$, and then assayed for component-C1 haemolytic activity. Full experimental details are given in the text. ○, Percentage inhibition of lysis of sensitized erythrocytes used in the component-C1 haemolytic assay, produced by the addition of rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q) to fibroblast culture medium; □, percentage inhibition of lysis of sensitized erythrocytes used in the component-C1 haemolytic assay, produced by the addition of rabbit fragment $F(ab')_2$ anti-(human subcomponent C1q) to human serum; Δ, percentage inhibition of lysis of sensitized erythrocytes used in the component-C1 haemolytic assay, produced by the addition of normal rabbit fragment $F(ab')_2$ to fibroblast culture medium or human serum.

Fig. 5. Inhibition of biosynthesis of component-C1 haemolytic activity by cycloheximide

Two identical cultures of fibroblasts were grown in parallel, and dilutions of the culture medium assayed for component-C1 haemolytic activity on days 0, 6, 8, 11 and 15. On day 6 the medium was removed and medium containing cycloheximide (200 µg/ml) was added to each culture. On day 8 the cycloheximide was removed from one of the cultures and fresh medium was added back to this culture. The monitoring of the release of component-C1 haemolytic activity by both cultures was continued. Δ, Percentage lysis of 1.5×10^8 sensitized erythrocytes used in the assay of component-C1 haemolytic activity, produced by a 1:20 dilution of the culture medium from the culture from which the cycloheximide had been removed on day 8; ○, percentage lysis of 1.5×10^8 sensitized erythrocytes used in the assay of component-C1 haemolytic activity, produced by a 1:20 dilution of the culture medium from the culture from which cycloheximide had not been removed.

functional C1 haemolytic activity from fibroblast culture medium.

(2) Incorporation of radioactivity into specific immunoprecipitates. This was performed by using anti-(subcomponent C1q), anti-(subcomponent C1r) and anti-(subcomponent C1s), as described in the Materials and Methods section. All the results quoted, which show the amount of radioactivity incorporated into an immunoprecipitate, are expressed as c.p.m. per total culture, since there were 2–3-fold differences in the amount of carrier protein added in different experiments. These differences in the amount of carrier protein added had no effect on the total radioactivity found in the final immunoprecipitate.

The DUV fibroblasts when labelled in the presence of culture medium containing 10% (v/v) heat-inactivated foetal calf serum yielded approx. 4000 c.p.m. at 6 h and 45000 c.p.m. at 72 h in immunoprecipitates made from the concentrated culture

medium after addition of carrier subcomponent C1q and anti-(subcomponent C1q). The immunoprecipitates made after adding carrier subcomponents C1r and C1s, and the appropriate antisera, contained approx. 2000 c.p.m. at 6 h and 20000 c.p.m. at 72 h.

The LNSV-40 fibroblasts when labelled in the presence of culture medium containing 10% (v/v) heat-inactivated foetal calf serum yielded 33000 c.p.m. at 72 h in immunoprecipitates made after adding carrier and anti-(subcomponent C1q), and 60000 c.p.m. at 72 h in immunoprecipitates made after adding carrier and antisera to subcomponents C1r and C1s. When LNSV-40 fibroblasts were labelled in serum-free culture medium (which also lacked the unlabelled amino acids corresponding to the labelled ones added) for 3 h, the incorporation of radioactivity into the immunoprecipitates was variable and ranged from 10000 to 200000 c.p.m. per culture in the subcomponent-C1q immunoprecipitates and from 13000 to 60000 c.p.m. per culture in the subcomponent-C1r and -C1s combined immunoprecipitates.

The labelling patterns found on SDS/polyacrylamide gels were similar when the patterns obtained for immunoprecipitates made from 3 h and 72 h radioactively labelled media were compared.

When radioactively labelled immunoprecipitates made by using anti-(subcomponent C1q) were run on 5.6% polyacrylamide gels in SDS without reduction of the disulphide bonds, no major radioactive peaks entered the gel. The unlabelled carrier subcomponent C1q readily entered the gel and was found in the expected positions [two peaks of apparent mol.wts. 69000 and 54000 in a ratio of 2:1, w/w; the molecular weights were calculated as described by Reid *et al.* (1972) and Reid & Porter (1976)] (Fig. 6a). When the non-reduced labelled subcomponent-C1q immunoprecipitates were run on 4% polyacrylamide gels in SDS, no major radioactive peaks were found in the sliced gel. When radioactively labelled subcomponent-C1q immunoprecipitates were reduced and alkylated and run on 5.6% polyacrylamide gels, most preparations showed either two major radioactive peaks with apparent mol.wts. of 47000 and 42000, or one major peak of apparent mol.wt. 47000, which had a distinct 'shoulder' in the lower-molecular-weight region (Fig. 6b). These radioactive peaks clearly separated from the heavy chain of IgG (52000 apparent mol.wt.) or the A chain of subcomponent C1q (34000 apparent mol.wt. in polyacrylamide gels run in SDS). A third, smaller, peak of radioactivity (22000 apparent mol.wt.) was usually observed, and this was separated from the C chain of subcomponent C1q, which has an apparent mol.wt. of 27000.

The profiles of 5.6% polyacrylamide gels run in SDS of immunoprecipitates made by using a mixture of antisera to subcomponents C1r and C1s

Fig. 6. Scans of polyacrylamide-gel electrophoresis in SDS of unreduced and reduced and alkylated subcomponent-C1q immunoprecipitates labelled with radioactivity

Immunoprecipitates were obtained from radioactively labelled fibroblast culture medium after the addition of carrier subcomponent C1q and anti-(subcomponent C1q). The samples were run on 5.6% polyacrylamide gels in the presence of SDS. —, Intensity of Coomassie Brilliant Blue stain (protein); ○, radioactivity (c.p.m.). (a) Unreduced subcomponent-C1q immunoprecipitate; (b) reduced and alkylated subcomponent-C1q immunoprecipitate. The marker proteins shown by the protein stain and indicated on the scale of apparent molecular weights are: for (a) IgG (mol.wt. 155000), subcomponent C1q (A-B dimer) (mol.wt. 69000), subcomponent C1q (C-C dimer) (mol.wt. 54000); for (b) heavy chain of IgG (mol.wt. 52000), A chain of subcomponent C1q (mol.wt. 34000), B chain of subcomponent C1q (mol.wt. 31600), C chain of subcomponent C1q (mol.wt. 27000), light chain of IgG (mol.wt. 23500).

Fig. 7. Scans of polyacrylamide-gel electrophoresis in SDS of unreduced and reduced and alkylated subcomponent C1r-C1s immunoprecipitates labelled with radioactivity

Immunoprecipitates were obtained from radioactively labelled fibroblast culture medium after addition of carrier subcomponents C1r and C1s and antisera to these subcomponents. Subcomponent C1q is also found in these immunoprecipitates, for the reasons discussed in the text. The samples were run on 5.6% polyacrylamide gels in the presence of SDS. —, Intensity of Coomassie Brilliant Blue stain (protein); ○, radioactivity (c.p.m.). (a) Unreduced subcomponent C1r plus C1s immunoprecipitate; (b) reduced and alkylated C1r-C1s immunoprecipitate. The marker proteins shown by the protein stain and indicated on the scale of apparent molecular weights are: for (a) 155 000, IgG; 98 000, subcomponent C1r; 92 000, subcomponent C1s; 69 000, subcomponent C1q (A-B dimer); 54 000, subcomponent C1q (C-C dimer); for (b) 83 000, subcomponents C1r and C1s; 52 000, heavy chain of IgG; 34 000, A chain of subcomponent C1q; 31 600, B chain of subcomponent C1q; 27 000, C chain of subcomponent C1q; 23 500, light chain of IgG.

are shown in Figs. 7(a) and 7(b). When the immunoprecipitation was performed in the presence of EDTA, and at 4°C, the subcomponents were not activated and accordingly displayed the very characteristic banding patterns that have been observed when the non-reduced (Fig. 7a) and reduced and alkylated (Fig. 7b) proenzyme forms of the subcomponents are run on polyacrylamide gels in SDS (Gigli *et al.*, 1976). The peaks of radioactivity found in the unreduced labelled sample were exactly coincident with the unreduced subcomponent C1r (apparent mol.wt. 98000) and unreduced subcomponent C1s (apparent mol.wt. 92000) (Fig. 7a). A single peak of radioactivity was found in the reduced and alkylated sample that ran with the marker reduced and alkylated proenzyme forms of subcomponents C1r and C1s, which both have an apparent mol.wt. of 83000 (Fig. 7b).

When immunoprecipitates of subcomponents C1r and C1s were made from culture medium that had been incubated at 37°C with antibody-antigen aggregates, and then made 10mM with respect to EDTA, the major radioactive peaks that entered the 5.6% polyacrylamide gel run in SDS followed the expected patterns obtained with the activated forms of subcomponents C1r and C1s. Under these conditions unreduced subcomponents C1r and C1s have apparent mol.wts. of 107000 and 92000

respectively (Sim *et al.*, 1977). Reduction of subcomponent C1r yields chains with apparent mol.wts. of 58000 and 36000, whereas reduced subcomponent C1s yields chains with apparent mol.wts. of 58000 and 29000 (Sim *et al.*, 1977). When the carrier subcomponents C1r and C1s were added in the form of human serum and activation was subsequently brought about during immunoprecipitation, then complexes between the inhibitor of the activated form of component C1 and subcomponents C1r and C1s were formed. These complexes are stable in polyacrylamide gels run in SDS (Harpel & Cooper, 1975), and the major radioactive peaks in these immunoprecipitates were coincident with the subcomponent-C1 inhibitor peaks.

When labelled medium was mixed with preformed anti-ovalbumin-albumin aggregates, in the presence of EDTA, radioactivity [amounting to approx. 60% of the radioactivity bound in anti-(subcomponent C1q) immunoprecipitates] was bound to the aggregates. When the labelled immune aggregates were reduced and alkylated and then examined on 5.6% polyacrylamide gels, run in SDS, there were three radioactively labelled peaks located in the 48000-38000 apparent-mol.wt. region, a labelled peak in the 155000 apparent-mol.wt. region and another labelled peak of very much higher molecular weight, which had just entered the top of

Fig. 8. Polyacrylamide-gel electrophoresis in SDS of reduced and alkylated radioactively labelled immune aggregates. Preformed immune aggregates of anti-ovalbumin and ovalbumin were incubated with radioactively labelled fibroblast culture medium in the presence of EDTA. The radioactivity bound to the immune aggregates was examined, after reduction and alkylation, on a 5.6% polyacrylamide gel run in SDS. Full experimental details are given in the text. The marker proteins whose position is indicated on the scale of apparent molecular weights are: 155000, IgG (added after reduction and alkylation of the sample); 52000, heavy chain of IgG; 23500, light chain of IgG.

the gel (Fig. 8). Without reduction and alkylation of the labelled immune aggregates, no major radioactively labelled peaks were seen when the sample was run on polyacrylamide gels in SDS.

Discussion

The results in the present paper clearly demonstrate that human fibroblast cell lines synthesize and secrete a haemolytic activity that is functionally identical in many respects with the component-C1 haemolytic activity found in human serum (Table 2; Figs. 2 and 3). This activity was found in the culture media of all the human fibroblast cell lines examined and was not detected in the culture media from a number of non-fibroblast cell lines (Table 1). Since the fibroblast-cell-line cultures are unlikely to contain significant amounts of any other type of cell, it appears probable that a single cell type may synthesize all the subcomponents of the component-C1 complex. However, the rate of synthesis and secretion of subcomponents C1r and C1s may be independent of the rate of synthesis and secretion of subcomponent C1q. Thus fibroblast culture media that had unexpectedly low amounts of the component-C1 haemolytic activity appeared to have normal amounts of the C1r and C1s subcomponents and a low subcomponent-C1q value, as judged by the increase in the component-C1 haemolytic activity caused by the addition of purified subcomponent C1q to the media with the low amounts of component-C1 haemolytic activity.

The ability to synthesize component-C1 haemolytic activity appears to be a general property of human fibroblasts and they, under the optimal experimental conditions obtained so far, synthesize and secrete this activity at a rate of approx. 10^4 effective component-C1 molecules per day *in vitro*. It is possible that, if this rate of synthesis and secretion takes place *in vivo*, fibroblasts may be a major source of the component-C1 haemolytic activity found in plasma, since they are one of the most abundant cell types in the body. Al-Adnani & McGee (1976) have shown that rat skin fibroblasts *in vitro* synthesized material that was antigenically similar to subcomponent C1q. They also presented evidence that this material was synthesized by the rat fibroblasts *in vivo*, at least in pathological conditions where the fibroblasts were functioning at a much higher rate than normal. Attempts to prepare primary cultures of specialized cell types are often hampered by the tendency of fibroblasts to grow more rapidly than other cells; therefore it appears that care would have to be taken to exclude fibroblasts from a cell culture if a study of the possible biosynthesis of component-C1 haemolytic activity by non-fibroblast cell lines is to be made.

Functionally and physiochemically, the fibro-

blast cell lines produce proteins that appear to be identical with the C1r and C1s subcomponents found in human serum. On the other hand, the protein synthesized by the fibroblasts that appears to fulfil the role of subcomponent C1q differs in certain respects from the subcomponent C1q found in normal human serum. The principal role of subcomponent C1q, of binding to immune aggregates, appears to be common to the fibroblast subcomponent C1q and subcomponent C1q purified from human serum. This is shown by the fact that the component-C1 haemolytic activity released by fibroblasts binds to erythrocytes sensitized with antibody (Table 2) and to insoluble antibody-antigen aggregates (Fig. 3). The binding, or fixation, of fibroblast component C1 and human serum component C1 appears almost identical in terms of haemolytic units bound per μg of antibody-antigen aggregate (Fig. 3), which suggests that they are bound by the same mechanism. However, the proposed fibroblast subcomponent C1q differs markedly, in at least two respects, from subcomponent C1q purified from human serum: (1) it appears to react very weakly with antisera prepared against subcomponent C1q purified from human serum (Fig. 4); (2) its apparent molecular weight, as judged by polyacrylamide-gel electrophoresis in SDS, is greater than that of purified subcomponent C1q (Figs. 6a, 6b and 8).

The interaction between rabbit fragment F(ab')_2 anti-(subcomponent C1q) and fibroblast or serum subcomponent C1q was examined by determining the ability of the antibody to inhibit the component-C1 haemolytic activity found in fibroblast culture medium or human serum (Fig. 4). Even when approximately one-fifth the amount of fibroblast component C1 compared with serum component C1 was used (on a haemolytic basis), there was a requirement for 30 times as much antibody to give 95–100% inhibition of the fibroblast component-C1 haemolytic activity compared with that required to give the same degree of inhibition of the serum component-C1 haemolytic activity (Fig. 4). This result can be interpreted in two ways: (1) that fibroblast subcomponent C1q is bound to fibroblast subcomponents C1r and C1s in such a way that it is not readily available to the antibody [it is noteworthy in this context that serum component C1, bound to antibody on a cell surface, in which the subcomponents C1q, C1r and C1s are known to be in a tight complex, is not so readily inactivated by anti-(subcomponent C1q) as it is in free solution (K. B. M. Reid & I. S. Todd, unpublished work)]; (2) that there are 'extra' polypeptide, or carbohydrate, extensions on the fibroblast subcomponent-C1q molecule that obscure the principal antigenic sites against which the anti-(subcomponent C1q) is directed. The studies involving electrophoresis of immunoprecipitates on polyacrylamide gels in SDS (Figs. 6a, 6b and 8) support

the view that fibroblast subcomponent C1q contains peptide extensions in all three of its chains and that these extensions are cross-linked by interchain disulphide bonds. With reduction and alkylation of disulphide bonds, the major portion of the radioactivity precipitated along with the subcomponent-C1q immunoprecipitate (Fig. 6b), or with immune aggregates in the presence of EDTA (Fig. 8), was found in polypeptides in the apparent mol.wt. range 48000–42000. The reduced and alkylated A, B and C chains of purified subcomponent C1q behave anomalously on polyacrylamide-gel electrophoresis in SDS, since they have apparent mol.wts. of 34000, 32000 and 28000 respectively as judged by this method (Reid *et al.*, 1972), whereas their true molecular weights are all in the range 22000–24000 (Reid & Porter, 1976). Therefore it is possible that the true molecular weights of the polypeptide chains of the radioactively labelled fibroblast material, if it is related to serum subcomponent C1q, may also be lower, i.e. in the range 36000–34000. Gel-filtration studies in 6M-guanidinium chloride should clarify this point. Assuming that the radioactively labelled material is a precursor form of the subcomponent C1q found in serum, it is possible that such a molecule would have A, B and C chains that were each approx. 12000 higher in molecular weight than that found in the serum form. Since there are 18 chains in subcomponent C1q, this would give an increase in molecular weight of 216000 for the entire molecule. If this is the case, then it would not be surprising that non-reduced fibroblast subcomponent C1q did not enter 5.6% or 4% polyacrylamide gels run in SDS (Fig. 6a). The presence of only two types of disulphide bond in the proposed extensions to the A, B and C chains (i.e. a B-to-C-type disulphide bond and an A-to-A-type disulphide bond) would yield a molecule of at least $21600 + 410000 = 626000$ molecular weight even under dissociating conditions.

It would not be entirely unexpected for subcomponent C1q to be synthesized as a precursor with disulphide-linked polypeptide extensions, since it is now well established that the three non-disulphide-linked polypeptide chains of certain collagen molecules are first synthesized as procollagen molecules with disulphide-linked polypeptide extensions at either the N-terminus or C-terminus of their chains (Nowack *et al.*, 1976; Olsen *et al.*, 1976; Byers *et al.*, 1975; Fessler *et al.*, 1975). Thus subcomponent C1q may show some relationship to collagen in the manner in which it is synthesized, which would be consistent with previous results that show that collagen-like regions are found in the A, B and C chains of subcomponent C1q, and that these regions contain hydroxylated amino acid residues, glycosylated hydroxylysine residues and probably form a typical collagen-like triple-helical structure (Reid,

1974, 1976; Calcott & Müller-Eberhard, 1972; Brodsky-Doyle *et al.*, 1976).

In conclusion, a component-C1-like haemolytic activity appears to be synthesized and secreted by human fibroblasts, and material functionally equivalent to each of the subcomponents C1q, C1r and C1s has been found. Identification of newly synthesized radioactively labelled precursor forms of subcomponents C1r and C1s has been made antigenically, by immunoprecipitation, and physicochemically, by electrophoresis on polyacrylamide gels run in SDS. Formal identification of newly synthesized radiolabelled subcomponent-C1q molecules in fibroblast culture media has proved difficult, since antigenically and physiochemically the radioactive material tentatively identified as fibroblast subcomponent C1q appears to differ from the subcomponent C1q found in normal serum.

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References

- Al-Adnani, M. S. & McGee, J. O'D. (1976) *Nature (London)* **263**, 145–146
- Bing, D. H., Spurlock, S. E. & Bern, M. M. (1975) *Clin. Immunol. Immunopathol.* **4**, 341–351
- Bøyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21**, Suppl. 97
- Brodsky-Doyle, B., Leonard, K. R. & Reid, K. B. M. (1976) *Biochem. J.* **159**, 279–286
- Byers, P. H., Click, E. M., Harper, E. & Bornstein, P. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3009–3013
- Calcott, M. A. & Müller-Eberhard, H. J. (1972) *Biochemistry* **11**, 3443–3450
- Colten, H. R. (1976) *Adv. Immunol.* **22**, 67–118
- Colten, H. R., Borsos, T. & Rapp, H. J. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1158–1163
- Colten, H. R., Gorden, J. M., Rapp, H. J. & Borsos, T. (1968) *J. Immunol.* **100**, 788–792
- Croce, C. M., Girardi, A. J. & Koprowski, H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3617–3620
- Day, N. K., Gewurz, H., Pickering, R. J. & Good, R. A. (1970) *J. Immunol.* **104**, 1316–1319
- Fairbanks, G., Stech, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Fessler, L. I., Morris, N. P. & Fessler, J. H. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4905–4909
- Gigli, I., Porter, R. R. & Sim, R. B. (1976) *Biochem. J.* **157**, 541–548
- Goodfellow, P., Barnstable, C., Jones, E., Bodmer, W. F., Crompton, M. J. & Snary, D. (1976) *Tissue Antigens* **7**, 105–117
- Harpel, P. C. & Cooper, N. R. (1975) *J. Clin. Invest.* **55**, 593–604
- Klein, E., Klein, G., Nadkarni, J. S., Nadkarni, S., Wigzell, H. & Clifford, P. (1967) *Lancet* **ii**, 1068–1070
- Lepow, I. H., Naff, G. B., Todd, E. W., Pensky, J. & Hinz, C. F. (1963) *J. Exp. Med.* **117**, 938–1008
- Minowada, J., Ohnuma, T. & Moore, G. E. (1972) *J. Natl. Cancer Inst.* **49**, 891–895

- Nichols, W. W., Murphy, D. G., Cristofalo, V. J., Toji, L. H., Greene, A. E. & Dwight, S. A. (1977) *Science* **196**, 60–63
- Nowack, H., Olsen, B. R. & Timpl, R. (1976) *Eur. J. Biochem.* **70**, 205–216
- Olsen, B. R., Hoffmann, H. P. & Prockop, D. J. (1976) *Arch. Biochem. Biophys.* **175**, 341–350
- Reid, K. B. M. (1971) *Immunology* **20**, 649–658
- Reid, K. B. M. (1974) *Biochem. J.* **141**, 189–203
- Reid, K. B. M. (1976) *Biochem. J.* **155**, 5–17
- Reid, K. B. M. & Porter, R. R. (1976) *Biochem. J.* **155**, 19–23
- Reid, K. B. M., Lowe, D. M. & Porter, R. R. (1972) *Biochem. J.* **130**, 749–763
- Reid, K. B. M., Sim, R. B. & Faiers, A. P. (1977) *Biochem. J.* **161**, 239–245
- Sim, R. B., Porter, R. R., Reid, K. B. M. & Gigli, I. (1977) *Biochem. J.* **163**, 219–227
- Solomon, E., Bobrow, M., Goodfellow, P. N., Bodmer, W. F., Swallow, D. M., Povey, S. & Noël, B. (1976) *Somatic Cell Genet.* **2**, 125–140
- Stecher, V. J., Morris, J. H. & Thorbecke, G. J. (1967) *Proc. Soc. Exp. Biol. Med.* **124**, 433–438
- Szybalski, W., Szybalski, E. H. & Ragni, G. (1962) *Natl. Cancer Inst. Monogr.* **7**, 75–89
- Tumilowicz, J., Nichols, W., Cholon, J. & Green, A. (1970) *Cancer Res.* **30**, 2110–2118
- Watkins, J. F. & Sanger, C. (1977) *Br. J. Cancer* **35**, 785–794