*Proc. Natl. Acad. Sci. USA*
Vol. 78, No. 12, pp. 7707–7711, December 1981
Immunology

# Induction of immune cytolysis: Tumor-cell killing by complement is initiated by covalent complex of monoclonal antibody and stable C3/C5 convertase

(cobra venom factor/alternative pathway/human melanoma cells/hybrid proteins)

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ABSTRACT **The unique specificity of monoclonal antibodies (Ab) was combined with the cell-killing function of the cytolytic alternative pathway of complement. The functionally C3b-like glycoprotein of cobra venom was linked to a murine monoclonal Ab directed to a human melanoma-associated antigen by a disulfide bond, by using a heterobifunctional crosslinking reagent. The covalent monoclonal Ab–cobra venom factor (CVF) complex exhibited specific cytolytic activity in the presence of normal or C4-deficient serum. It induced killing of melanoma cells but not of LG-2 lymphoblastoid cells or P815 mastocytoma cells. The cytolytic action of the monoclonal Ab–CVF complex was selective in that it was limited to the melanoma cells when these were mixed with one of the two other cell lines. In absence of serum, the complex was noncytotoxic. Monoclonal Ab or CVF alone had no cytolytic activity with or without serum. It is concluded that CVF covalently linked to melanoma cell-bound Ab forms the stable C3/C5 convertase with factors B and D of the alternative pathway, which in turn causes formation of the membrane attack complex and thereby cell death.**

We describe here an immunocytolytic complex that consists of a monoclonal antibody (Ab) and the C3b-like glycoprotein of cobra venom (CVF). Itself nontoxic, the complex becomes cytolytic in the presence of serum complement.

The uniquely specific monoclonal Abs complexed with cytotoxic substances may become effective means of selectively eliminating defined cell populations, including tumor cells with tumor-associated antigens. Various approaches have been taken involving the covalent coupling of diphtheria toxin (1), ricin (2), or gelonin (3) to antibody molecules. From a conceptual point of view it appeared ideal to us to render a monoclonal Ab capable of utilizing one of the host's own cytotoxic mechanisms—for instance, complement. Such an approach should circumvent nonspecific toxicity and the need for internalization of the antibody conjugate by the target cells to become effective (4).

We therefore coupled CVF, the structural subunit of a C3/C5 convertase of the alternative complement pathway (5), to monoclonal murine Ab. CVF appears to be immunochemically (6) and structurally (7) related to human C3. It exhibits a C3b-like function when added to mammalian serum in that, together with factor B and in presence of factor D and $Mg^{2+}$, it forms a soluble C3/C5 convertase (8) that efficiently initiates assembly of the membrane attack complex (MAC) of complement (9). Unlike C3b, which forms a labile convertase with factor B (C3b,Bb), CVF forms an unusually stable enzyme (CVF,Bb) (10) which, in contradistinction to C3b,Bb, is resistant to inactivation by the complement control proteins, factors H and I (11). Because of these unusual functional characteristics, CVF was chosen for the construction of the Ab–effector molecule complex rather than C3b.

In the following it will be shown that the Ab–CVF complex prepared with monoclonal Ab to a melanoma-associated antigen (12) exhibits target cell-specific cytotoxicity *in vitro* in the presence of serum.

## MATERIALS AND METHODS

**Materials.** CVF was isolated from lyophilized cobra venom (International Biological Extracts) as described (13). Cell lines of human melanoma (M21), human lymphoblastoma (LG-2), and mouse mastocytoma (P815) were maintained in continuous culture as described (14). The IgG2a hybridoma antibody 9.2.27 to a 250,000-dalton melanoma-associated antigen was made available by R. A. Reisfeld (12). The monoclonal Ab was isolated from ascites of BALB/c mice by affinity chromatography on protein A-Sepharose 4B-Cl (Pharmacia). The production of a rabbit xenoantiserum to intact melanoma cells has been described (15). Normal guinea pig serum was purchased (Cordis Laboratories) and C4-deficient serum was obtained from C4-deficient guinea pigs. Molecular weight marker proteins were purchased from Bio-Rad.

**Formation of Disulfide-Linked Complexes of CVF and Monoclonal Ab.** To a solution of either protein in 0.1 M sodium phosphate/0.1 M NaCl, pH 7.5, 20 mM *N*-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia) in absolute ethanol was added to give a 5.5 molar excess. The mixture was stirred at 20°C for 30 min and filtered on a column of Sephadex G-25 medium (Pharmacia) to remove released N-hydroxysuccinimide and excess reagent. The average number of 2-pyridyldithiopropionyl groups introduced per molecule of protein was calculated as described (16). 2-Pyridyldithiopropionylated CVF was radiolabeled with $^{125}I$ (New England Nuclear) by using Iodogen (Pierce) (17). To a solution of 2-pyridyldithiopropionylated Ab in 0.1 M sodium acetate/0.1 M NaCl, pH 4.5, 500 mM dithiothreitol was added to give a final concentration of 45 mM. The mixture was stirred at 20°C for 20 min and then filtered on Sephadex G-25 to remove released pyridine-2-thione and excess dithiothreitol. The thiol group-containing Ab was immediately used for the coupling reaction. Equimolar amounts of 2-pyridyldithiopropionylated $^{125}I$-labeled CVF ($^{125}I$-CVF) and thiolated Ab were mixed, kept at 20°C for 21 hr in 3 ml at 1.5 mg of protein per ml, and then filtered at 4°C on a column (1.6 × 56 cm) of Ultrogel AcA 22 (LKB); 1.2-ml fractions were collected at a linear flow rate of 2 cm/hr to separate released pyridine-2-thione and nonreacted $^{125}I$-CVF and Ab from disulfide-linked complexes of $^{125}I$-CVF and Ab.

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Abbreviations: Ab, antibody; CVF, cobra venom factor; MAC, membrane attack complex.

**$^{51}$Cr-Release Cytotoxicity Assay.** Five million viable cells in a total volume of 450 μl were labeled with 200 μCi (1 Ci = 3.7 × $10^{10}$ becquerels) of $Na_2{}^{51}CrO_4$ (New England Nuclear) for 1 hr at 37°C. Cells were washed once, incubated for 1 hr at 37°C, washed three times, and adjusted to 5000 cpm/50 μl of suspension, which corresponded to 1 × $10^5$ M21 cells per ml, 2 × $10^5$ LG-2 cells per ml, and 4 × $10^5$ P815 cells per ml. Duplicate 50-μl cell suspension samples were incubated for 30 min at 37°C with 50 μl of monoclonal Ab–CVF complex. The cells were sedimented, the supernatant was discarded, 150 μl of guinea pig serum was added, and the cell suspension was incubated for 5 hr at 37°C in 5% $CO_2$/95% air in a humidified chamber. The reaction was stopped by adding 700 μl of cold buffer (10 mM sodium phosphate/140 mM NaCl/0.5% bovine serum albumin, pH 7.5), vortexing, and centrifugation. An aliquot of the supernatant was assayed for radioactivity. Controls consisted of polyclonal antiserum, monoclonal Ab, CVF, buffer, or culture medium. Specific $^{51}$Cr release was calculated as:

$$\frac{100 \times (\text{experimental cpm} - \text{spontaneous cpm})}{(\text{maximal cpm} - \text{spontaneous cpm})}.$$

**Scanning Electron Microscopy.** M21 cells (2.3 × $10^6$) were placed on round glass coverslips coated with poly(L-lysine) (Vega Biochemicals) in flat-bottom wells of a tissue culture dish. Adhering cells were preincubated with either monoclonal Ab–CVF complex at 50 μg/ml, polyclonal antiserum, or culture medium and were subsequently treated with guinea pig serum as described above. After 2.5 hr of incubation, the cells were fixed for 30 min with 2% glutaraldehyde/0.1 M sodium cacodylate, pH 7.2, stained for 1 hr with 1% $OsO_4$ in cacodylate buffer, dehydrated, and subjected to critical point drying with monochlorotrifluoromethane (Matheson). After sputter coating with carbon, the samples were examined with a Hitachi S-500 scanning electron microscope at 20 kV.

## RESULTS

**Preparation, Isolation, and Demonstration of Covalent Complexes of Monoclonal Ab and CVF.** The derivatization of CVF and the monoclonal Ab to melanoma-associated antigen resulted in an average of 3.0 2-pyridyldithiopropionyl groups per CVF molecule and of 2.6 groups per Ab molecule. Assuming random attachment of 2-pyridyldithiopropionyl groups, it was calculated according to Poisson distribution that only 5% of the CVF and 7.4% of the Ab molecules remained unmodified.

After transformation of 2-pyridyldithiopropionyl groups to free thiol groups in the Ab molecules, the coupling reaction was performed. As shown by gradient gel electrophoresis (Fig. 1), several products of high molecular weight were formed which constituted predominantly dimers, trimers, and tetramers. Because of the chemical coupling reaction used, only hybrid complexes could be formed: dimers, Ab,CVF; trimers, $Ab_2$,CVF or Ab,$CVF_2$; and tetramers, $Ab_2$,$CVF_2$, $Ab_3$,CVF, or Ab,$CVF_3$. Under reducing conditions the complexes of CVF and Ab completely dissociated, resulting in a mixture of the α, β, and γ chains of CVF and the heavy and light chains of the Ab. The mixture of unreduced proteins was subjected to gel filtration to separate the monoclonal Ab–CVF complexes from monomeric CVF and Ab (Fig. 2). The monoclonal Ab–CVF complexes were found to be stable for several months at 4°C. Two separate preparations were made and had comparable properties.

**Killing of Melanoma Cells by Covalent Complexes of Monoclonal Ab and CVF in Presence of Normal and C4-Deficient Serum.** Complexes of monoclonal Ab and CVF were able to induce cytotoxic activity in serum against M21 melanoma cells. Specific $^{51}$Cr release from the target cells reached 65% within 5 hr (Fig. 3). A 5-μg dose of complex was at least as efficient

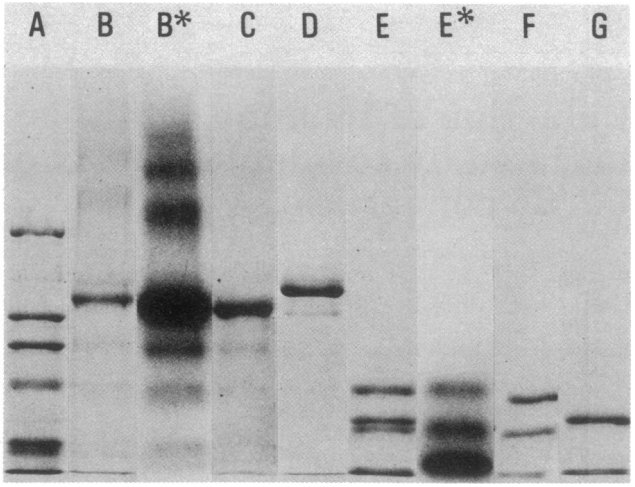


FIG. 1. Demonstration of disulfide-linked monoclonal Ab–CVF complexes by $NaDodSO_4$ gradient polyacrylamide gel electrophoresis. Lanes: A, molecular weight markers (from top: myosin, 200,000; β-galactosidase, 116,500; phosphorylase B, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000); B, monoclonal Ab–CVF complexes and unconjugated protein; B*, autoradiograph of B, showing distribution of $^{125}$I-CVF; C, $^{125}$I-CVF; D, monoclonal Ab; E, dithiothreitol-reduced B; E*, autoradiograph of E, showing distribution of α, β, and γ chains of $^{125}$I-CVF; F, reduced C, showing the three-chain composition of CVF; G, reduced D, showing position of murine IgG heavy and light chains.

in killing the melanoma cells as 50 μl of a polyclonal unadsorbed antiserum raised against whole melanoma cells. In contrast, the unconjugated monoclonal Ab tested in amounts of 2.5–100 μg showed no killing activity, presumably because it was incapable of activating complement by itself. CVF tested by itself in amounts of 2.5–35 μg or a mixture of CVF and monoclonal Ab in presence of serum as inactive. Spontaneous release of $^{51}$Cr from melanoma cells showed no difference in presence of serum or culture medium, indicating that M21 melanoma cells do not activate the alternative pathway of guinea pig complement.

Fig. 2 suggests that monoclonal Ab–CVF complexes of higher molecular weight are more efficient in causing cytolysis than are those of lower molecular weight. That the Ab–CVF complex

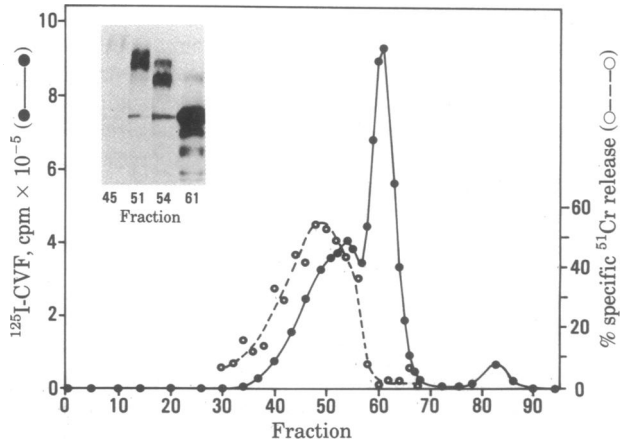


FIG. 2. Isolation of monoclonal Ab–CVF complexes by gel filtration (Ultrogel AcA 22). Shown is the elution profile of the reaction mixture containing the monoclonal Ab–$^{125}$I-CVF complexes (—●——●—). (*Inset*) Autoradiographs, after gradient gel electrophoresis, of fraction 61 (remaining monomeric CVF), fraction 54 (dimers), fraction 51 (trimers), and fraction 45 (higher oligomers). The distribution of cytolytic activity for melanoma cells is indicated (—○- -○—).

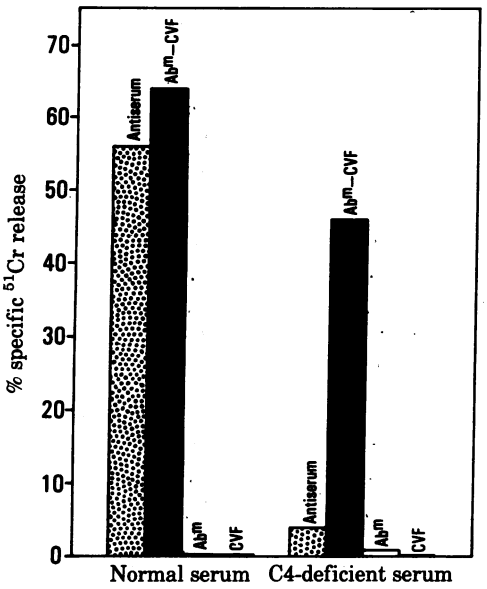


FIG. 3. Killing of M21 melanoma cells by the monoclonal Ab–CVF complex (Ab$^m$-CVF) utilizing the alternative pathway of complement. Five micrograms of complex or 50 μl of undiluted antiserum to intact melanoma cells was assayed for killing activity in presence of normal (*Left*) or C4-deficient (*Right*) guinea pig serum. The antiserum was active only in presence of normal serum; the monoclonal Ab–CVF complex was active in C4-deficient serum as well, indicating the involvement of alternative pathway proteins.

exerts its cytolytic effect by utilizing alternative pathway proteins is evident from the ability of C4-deficient serum to provide the necessary cytolytic ingredients (Fig. 3). The polyvalent antiserum to the melanoma cells was nonlytic in absence of C4. Fig. 4 shows that killing of melanoma cells by the monoclonal Ab–CVF complex is dependent on dose. Fig. 5 depicts the kinetics of the killing reaction, showing that the reaction reaches its plateau at 6 hr.

Fig. 6 illustrates the cytotoxic effect of the monoclonal Ab–CVF complex in presence of serum as seen by scanning electron microscopy. After treatment of M21 melanoma cells with the monoclonal Ab–CVF complex and complement, their appearance changed: the number of surface protrusions decreased, clusters of erosions appeared, occasionally large membrane blebs were seen, and, finally, membrane disintegration occurred.

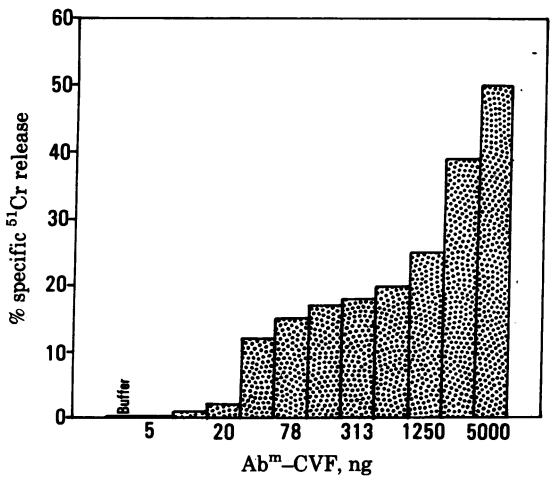


FIG. 4. Dependence of melanoma cell killing on the dose of the monoclonal Ab–CVF complex.

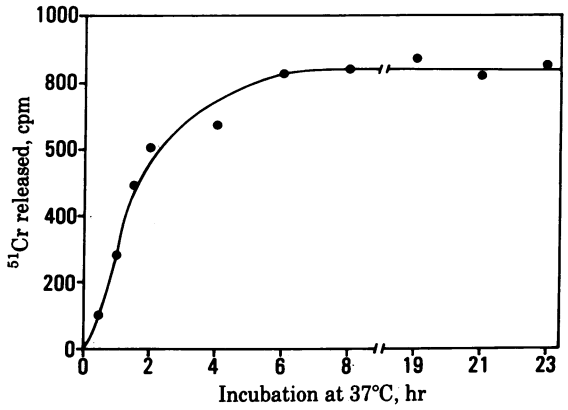


FIG. 5. Time course of M21 melanoma cell killing by monoclonal Ab–CVF complex. Samples of M21 melanoma cells were treated with 625 ng of monoclonal Ab–CVF complex (Ab$^m$-CVF) and serum or phosphate buffer and serum. $^{51}$Cr release: cpm in presence of Ab$^m$-CVF minus cpm in presence of buffer.

**Specific and Selective Cytotoxic Effect of the Monoclonal Ab–CVF Complex.** The monoclonal Ab used in this study appears to be specific for a melanoma-associated antigen (12). Although it binds to different melanoma cell lines, it does not react with certain other human tumor or normal cells. To demonstrate the specificity of the monoclonal Ab–CVF complex, we tested its cytotoxic effect on a human lymphoblastoid cell line (LG-2) and a mouse mastocytoma cell line (P815). Neither the monoclonal Ab–CVF complex nor the monoclonal antibody or CVF alone was able to induce lysis in presence of serum. In order to demonstrate selectivity of the monoclonal Ab–CVF complex, its cytolytic effect was examined in mixtures of M21 melanoma cells with either LG-2 lymphoblastoid cells or P815 mastocytoma cells (Figs. 7 and 8). While killing of melanoma cells proceeded as before, no bystander cell lysis was observed.

## DISCUSSION

The covalent complex of CVF and murine monoclonal Ab to a melanoma-associated antigen was shown to be an effective cytolytic tool *in vitro*: in the presence of serum, it killed M21 melanoma cells but not P815 mastocytoma cells and LG-2 lymphoblastoid cells. The cytotoxicity of the complex was selective when examined in a mixture of M21 and LG-2 or P815 cells.

The effector molecule chosen for transport by the monoclonal Ab is not cytotoxic *per se*. Instead, it combines in mammalian serum with factor B, the major proenzyme of the alternative complement pathway, to form, upon activation by factor D, the C3/C5 convertase, CVF,Bb (5, 8, 18). This enzyme is closely related to the physiological enzyme C3b,Bb (5), however, differs from it in several important respects. (*a*) Whereas the half-life of C3b,Bb at 37°C is 1.5 min (19), that of CVF,Bb is 7 hr (10, 18). (*b*) C3b,Bb is actively disassembled by factor H (20), and C3b is degraded and inactivated by the combined action of factors H and I (21); in contrast, CVF and the enzyme CVF,Bb are entirely resistant to the action of these complement control proteins (11). (*c*) C3b,Bb requires additional C3b in order to be able to act on C5 (19, 22); the CVF-dependent enzyme can activate C5 directly and thus initiate MAC formation without C3 (23, 24). (*d*) Whereas nascent or metastable C3b can attach itself firmly to targets of complement attack (5), CVF lacks such binding ability.

The alternative pathway of complement is activated by activator particles without antibody (25). It is the particle-bound C3b molecule that distinguishes between activators and non-

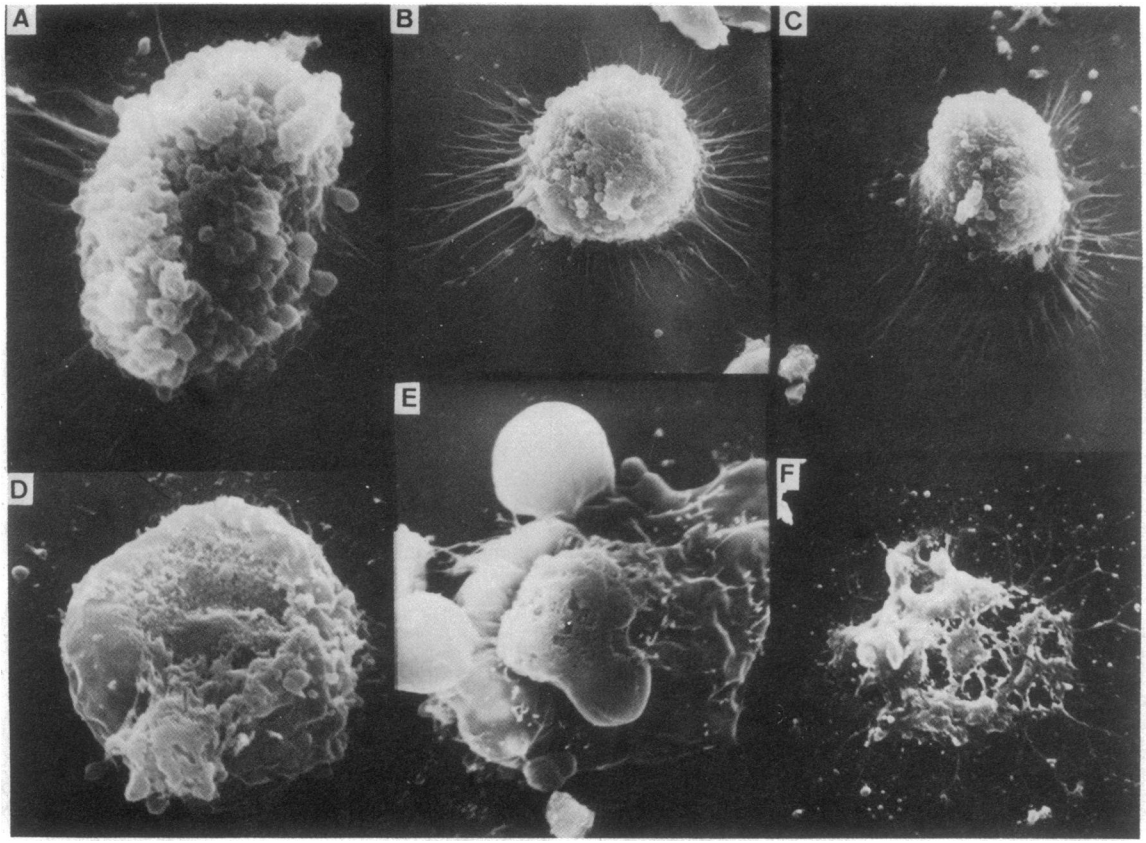


FIG. 6. Scanning electron microscopy of viable M21 melanoma cells before and after treatment with monoclonal Ab–CVF complex and complement. (*A–C*) Viable M21 melanoma. (*A*, ×4000; *B*, ×2000; *C*, ×2100.) (*D–F*) Treated M21 melanoma cells. (*D*, ×4000; *E*, ×3500; *F*, ×4000.)

activators of the pathway (26). Only on activators is it relatively restricted from control by factors H and I. Covalent linking of the control-resistant CVF to specific antibody directed to cell surface antigens brings the cytolytic alternative pathway to bear on any target cell regardless of whether or not it is an activator of the pathway. It is important to realize that assembly of the C3b- or CVF-dependent C3/C5 convertase occurs spontaneously because its activating enzyme, factor D, is always present in plasma in active form (27). These conditions do not pertain to the formation of the classical C3/C5 convertase because its activating enzyme, C1, occurs in plasma as an inactive proenzyme which requires activation by antibody aggregates. Thus, the synthetic monoclonal Ab–CVF complex constitutes the activator of a specific cytolytic alternative pathway that is inde-

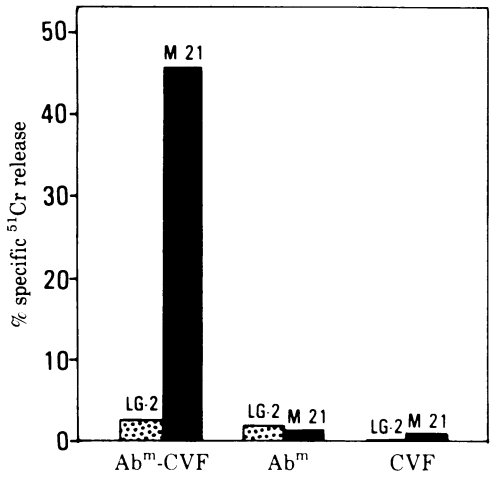


FIG. 7. Selective action of monoclonal Ab–CVF complex on M21 melanoma cells mixed with LG-2 lymphoblastoid cells. Duplicate samples of $^{51}$Cr-labeled M21 cells in the presence of a 1.5-fold excess of unlabeled LG-2 cells and unlabeled M21 cells in the presence of a 1.5-fold excess of $^{51}$Cr-labeled LG-2 cells were subjected to the cytotoxicity test with 5 μg of monoclonal Ab–CVF complex (Ab$^m$-CVF). Controls consisted of 2.5 μg of the monoclonal Ab (Ab$^m$) or of CVF.

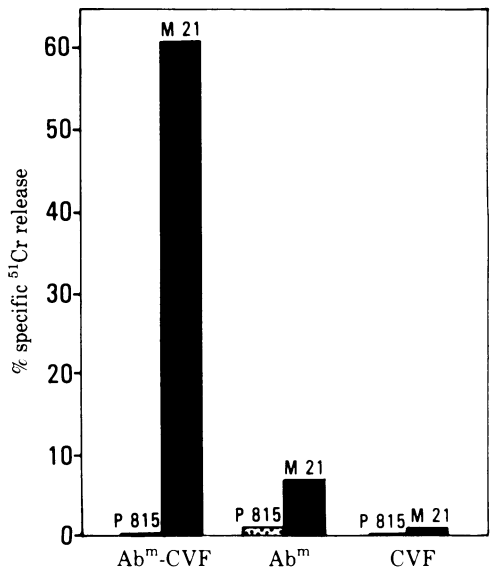


FIG. 8. Selective action of monoclonal Ab–CVF complex for M21 melanoma cells in mixture with P815 mastocytoma cells. The assay is described in the legend to Fig. 7.

pendent of the initial enzyme of either pathway and not subject to control by factors H and I.

The details of the reaction mechanism of the cytolytic complex remain to be analyzed. The rate of $^{51}Cr$ release from the melanoma cells, which was 66% of maximum at 3 hr, suggests that the cells defend themselves against MAC assault at least to some extent. When Raji cells were exposed to the isolated cytolytic alternative pathway in absence of antibody, they were killed with a half-time of 10 hr (28). The slow rate of killing was ascribed to their being weak activators of the alternative pathway and to their ability to defend themselves against low-rate MAC attack. However, when the same Raji cells were attacked rapidly, by using performed C5b-6 and C7, C8, and C9, cell death occurred within a few minutes (29). Those investigations indicated that death of nucleated cells is related to the extent and rate of MAC assault on the target.

The *in vivo* effect of the complex is not yet known. Radiolabeled CVF injected into rabbits has a plasma disappearance half-life of 32 hr (30). After approximately 4 days the animals developed antibodies that eliminated CVF from their circulation. It is likely that the anti-melanoma Ab–CVF complex localizes at its immunospecific target within a short time after injection into a tumor-bearing animal and that the localized complex not only exerts a cytotoxic effect but also produces localized inflammation by releasing the peptides C3a and C5a (31) and the fragments Bb (32) and C3b (5).

CVF-modified monoclonal Ab may become a useful tool for the selective elimination of cells that bear distinguishing antigenic markers. Whereas melanoma cells were selected for this study, other cell populations are likely to be similarly susceptible to selective killing. CVF is a three-polypeptide chain protein with a molecular weight of approximately 140,000. It may become possible to identify the submolecular regions that are essential for its function. Attempts at producing an active fragment of CVF or a synthetic peptide that subserves the CVF function but is less immunogenic may be warranted.

The authors thank Drs. Bumol and Reisfeld (Research Institute of Scripps Clinic) for supplying M21 melanoma cells, the monoclonal murine antibody 9.2.27, and the xenoantiserum to human melanoma cells. This is publication number 2551 from the Research Institute of Scripps Clinic. This research was supported by Grants AI 17354, CA 27489, and HL 16411 from the National Institutes of Health. C.-W.V. is the recipient of Research Fellowship Vo 303/1 from the Deutsche Forschungsgemeinschaft. H.J.M.-E. is the Cecil H. and Ida M. Green Investigator in Medical Research at the Research Institute of Scripps Clinic.

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