ent with microinvasive or invasive cancer. In contrast, 19 percent or less of the mice in the experimental group had cellular abnormalities consistent with dysplasia, and none of the animals had changes consistent with microinvasive or invasive cancer. The accuracy of the cytological interpretations is shown by comparing the cytological and histological findings. In the experimental group (Table 2), 15 percent of the mice had cellular evidence of dysplasia and this lesion was present in 20 percent of the histological specimens (Table 1). In the control group (Table 2), 83 percent of the animals had cellular evidence of dysplasia, microinvasive cancer, or invasive cancer, and these lesions were present in 91 percent of the histological specimens (Table 1). In micro-ELISA tests for HSV-2 antibody, the mean optical densities of sera from ten immunized and ten control mice were 0.554 and 0.070, respectively. The higher optical densities in sera from immunized mice indicate the presence of circulating antibody against HSV-2. The reaction in sera from control animals reflects nonspecific background activity.

In the control mice the yield of preinvasive and invasive lesions of the cervix was similar to that observed in previous studies of carcinogenesis with Formalin or ultraviolet-inactivated HSV-1 or HSV-2 (*3*, *4*). The duration of cervical exposure required for development of lesions was also similar to that observed previously. The experimental group developed no invasive lesions, and only 20 percent had dysplasia that persisted to the end of the study. Since an appreciable number of animals in both groups died early in the study from pneumonia or multiple abscesses and were unsuitable for histological analysis, the data in Table 2 are of particular importance. They show that, although many of the deaths in both groups occurred before 40 weeks of exposure had elapsed, the most advanced lesion at that time was microinvasive carcinoma which was present only in the control group. This is not a lethal lesion. In previous studies involving over 600 mice, frank invasive cancer was never observed before the 60th week of cervical exposure to HSV-2. Moreover, throughout the study none of the animals in the experimental group demonstrated cytological changes associated with microinvasive or frank invasive cancer. There was no cytological or gross evidence indicative of acute herpetic infection in any of the animals during the study. It is unlikely, therefore, that any of the early deaths resulted from cancer or acute herpetic infection.

Table 2. Cytological diagnoses in relation to duration of exposure in experimental and control mice. Values are numbers of mice.

Diagnosis	Length of exposure (weeks)				
	0	20	40	60	80
Experimental mice					
Negative	52	48	21	23	17
Dysplasia	0	0	5	3	3
Microinvasion	0	0	0	0	0
Invasion	0	0	0	0	0
Total	52	48	26	26	20
Control mice					
Negative	54	46	9	8	4
Dysplasia	0	0	18	15	8
Microinvasion	0	0	10	6	9
Invasion	0	0	0	0	2
Total	54	46	37	29	23

The detection of circulating antibody against HSV-2 in the inoculated animals and the absence of antibody in the control group indicate that the experimental mice were immunized before cervical exposure. Although the protection against the carcinogenic effects of HSV-2 is presumed to be of immunological origin, the presence of circulating antibody should not be construed as indicating the mechanism involved.

The data suggest that inoculation with ultraviolet-inactivated HSV-2 plus Freund's adjuvant conferred protection from the carcinogenic effect of HSV-2 on the cervical epithelium. Prevention of cervical cancer by this method provides additional support for the concept that HSV-2 has a carcinogenic action in the mouse genital tract. This model may be useful for further investigation of the possibility of vaccinating against cervical cancer.

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Monoclonal Antibodies to a Synthetic Fibrin-Like Peptide Bind to Human Fibrin but Not Fibrinogen

Abstract. *A synthetic heptapeptide from the amino terminus of the β chain in human fibrin was used as an antigen to produce monoclonal antibodies that bind to fibrin even in the presence of human fibrinogen at the concentration found in plasma. As expected, the antifibrin activity was inhibited by the peptide antigen but not by a control heptapeptide. In a chicken ex vivo circulatory model for fibrin detection, intravenously administered monoclonal antibodies bound to human fibrin–coated disks placed in an extracorporeal chamber. These findings may lead to better methods for identifiying deep vein and coronary artery thrombi.*

In man the detection and localization of deep vein and coronary artery thrombi are clinically important problems. The use of antibodies as agents for the immunodetection of fibrin deposits in vivo has been hampered by antifibrin cross-reactivity with fibrinogen, the precursor of fibrin. Day *et al.* (*1*) used ^{131}I-labeled antibodies to fibrin to determine the location of rat sarcomas. However, they and others (*2*) recognized the limitation of using antibodies to fibrin that cross-react with fibrinogen.

Blood clots form when thrombin cleaves two pairs of small peptides from fibrinogen to yield fibrin monomers (*3*). Fibrin monomers spontaneously aggregate into an insoluble gel, which is then covalently stabilized by Factor XIIIa. Despite the dramatic physical change, fibrin retains 98 percent of the original covalent structure of fibrinogen. Thus, antisera to fibrin cross-react strongly with fibrinogen; there has, to our knowledge, been only one isolated report of a fibrin-specific serum (*4*).

Since the fibrin molecule shares many epitopes with fibrinogen, we focused the immune response on a synthetic, fibrin-unique peptide. We anticipated that antibodies which recognized the synthetic fibrin epitope might bind to fibrin exclusive of fibrinogen (*5*). The heptapeptide of the amino terminus of fibrin's β chain was synthesized to serve as a fibrin-unique antigen, since we reasoned that the amino terminus is exposed after thrombin cleavage. Along with the first

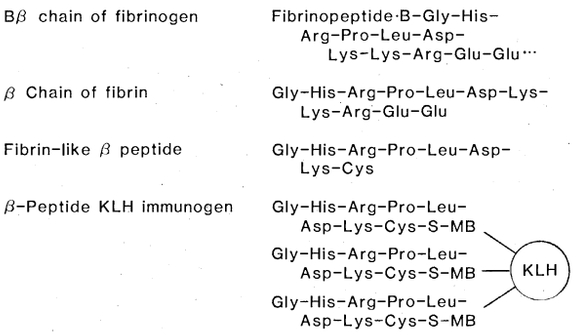

Fig. 1. Structure of fibrin-like β peptide and its conjugate with hemocyanin. Abbreviations: Gly, glycine; His, histidine; Arg, arginine; Pro, proline; Leu, leucine; Asp, aspartic acid; Lys, lysine; Cys, cysteine; Glu, glutamic acid; and S, serine.

seven amino acids of the β chain (Fig. 1), cysteine was placed at the carboxylterminus to permit unidirectional attachment of the synthetic peptide to maleimidobenzoylated keyhole limpet hemocyanin (MB-KLH). It was assumed that after the β peptide was reacted with MB-KLH all peptide amino termini would be uniformly oriented away from the protein carrier in a manner most analogous to that of fibrin β chains.

Using the Merrifield solid-phase method (*6*), we assembled the first seven amino acids of the fibrin β chain on Cys(3,4-dimethylbenzyl)-OCH_2 resin in a stepwise fashion. A sample of the completed peptidyl resin, Gly-His(tosyl)-Arg(tosyl)-Pro-Leu-Asp(benzyl)-Lys(2-chloro-benzyloxycarbonyl)-Cys(3,4-dimethylbenzyl)-OCH_2-resin, was evaluated by quantitative solid-phase Edman degradation. The resulting sequencing data confirmed that the desired amino acid sequence was present and indicated that the average level of amino acid deletions was less than 1 percent (*7*). After treatment with anhydrous hydrogen fluoride, the fully deprotected peptide gave the following amino acid composition on acid hydrolysis: Asp, 1.05; Pro, 1.01; Gly, 0.98; Leu, 1.01; His, 0.90; Lys, 1.03; and Arg, 1.02. The peptide was then coupled to MB-KLH (*8*) and the reaction was monitored by titrating the remaining sulfhydryl groups (*9*).

Hybridoma cell lines were established from a somatic cell fusion experiment (*10*) by using spleen cells from an immunized female BALB/c mouse and the Sp2/0 myeloma cell line. The donor mouse was one of three mice that had been immunized 10 weeks earlier with 30 μg of β-fibrin peptide–MB-KLH conjugate; a single booster injection was given intraperitoneally 4 weeks later. The plasma of each mouse contained antibodies that reacted with human fibrin monomer in the presence of fibrinogen at a concentration of 4 mg/ml. The mouse with the highest antifibrin response was chosen. For three consecutive days before the somatic cell fusion experiment, that mouse was hyperimmunized with conjugate (50 μg, half given intravenously and half intraperitoneally).

After 11 days in primary culture 13 wells contained cells that had multiplied to confluence. Portions of culture medium from those wells were withdrawn and mixed with heparin and phenylmethylsulfonyl fluoride. Thirty minutes after an equal volume of human fibrinogen (8 mg/ml) was mixed with the heparinized portion, the mixture was tested on microtiter wells coated with fibrin monomer (*11*). Antifibrin activity was detected in 3 of 13 wells; 7 of the remaining wells contained anti-KLH activity; none contained both activities. After 20 days in primary culture 12 of 72 confluent wells contained antifibrin immunoreactivity that was not inhibitable by fibrinogen. Seven cell lines were arbitrarily selected for cloning by a limiting dilution method, but only three continued to yield antifibrin-producing hybridomas. The stability and monoclonality of these three cell lines were demonstrated by recloning each line at limiting dilution; a high percentage (90 percent) of those resulting cell lines continued to yield fibrin-specific antibodies. The isoelectric points of the monoclonal antibodies, 55D10, 59D8, and 64C5, were 6.5, 6.4, and 7.6, respectively. The isotype of each monoclonal antibody was γ_1 for the heavy chains and κ for the light chains.

The screening method used above permitted selection of the monoclonal antibodies that bound to human fibrin monomer in the presence of fibrinogen. The binding to cross-linked human fibrin, that is, aggregated fibrin monomers, was then tested. Because clotted fibrin is mechanically difficult to manipulate quantitatively, 9.5-mm disks of Bunn coffee filter paper were used as solid supports. On each paper disk human fibrinogen (40 μg in 20 μl; Kabi grade L) was clotted by adding human thrombin (1.2 NIH units in 25 μl; Sigma) in the presence of 20 m*M* $CaCl_2$ and Trasylol [0.05 kallikrein-inhibiting units (KIU) per microliter; Mobay]. After 2 hours the fibrin-bearing disks were washed exhaustively and then stored in 10 m*M* KH_2PO_4 containing 0.15*M* NaCl and 0.02 percent NaN_3 (PBSA) in the presence of Trasylol (50 KIU/ml) at 4°C. By measuring the absorption of solutions before and after clotting at 280 nm we calculated the clotting efficiency at 80 percent. Fibrin disks were stained strongly with a solution of Coomassie Brilliant Blue if thrombin had been used in the clotting mixture.

Table 1. Inhibition of antibody binding to human fibrin by synthetic fibrin-like peptides. Direct binding to human fibrin disks was performed as described in the legend to Fig. 1, except a fibrin-like peptide at a final concentration of 0.5 mg/ml was used as an inhibitor. Gly-His-Arg-Pro-Leu-Asp-Lys-Cys was the structure of the β-chain peptide; the corresponding structure for the peptide from the amino terminus of the fibrin α chain was Gly-Pro-Arg-Val-Val-Glu-Arg-Cys. Maximum binding (100 percent) was determined for each monoclonal antibody in the absence of any inhibitor. All determinations were done in duplicate; values are means ± standard deviations.

Monoclonal antibody	Inhibition in presence of a fibrin-like peptide (percent)	
	β-Chain peptide	α-Chain peptide
55D10	82 ± 8.9	9 ± 6.3
59D8	92 ± 6.0	9.6 ± 1.3
64C5	87 ± 27	2.7 ± 5.0

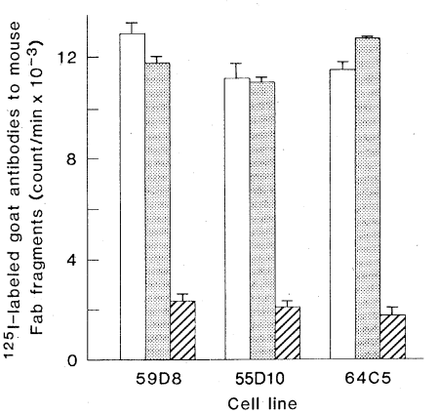

Fig. 2. Effect of fibrinogen on the direct binding of antibodies 59D8, 55D10, and 64C5 to fibrin-coated disks. For each monoclonal antibody, fibrin-bearing disks, prepared as described in text, were treated for 60 minutes with heparinized culture supernatant (50 μl) containing antibody in the absence (open bars) and presence (shaded bars) of fibrinogen (4 mg/ml). Culture medium (hatched bars) was used as a negative control. Specifically bound antibodies were detected by treatment with ^{125}I-labeled goat antibodies to mouse Fab′ fragments in 50 μl for 60 minutes. Each bar represents the mean ± standard deviation of two determinations made in parallel.

Recognition of aggregated fibrin by each monoclonal antibody was confirmed with the fibrin-coated disks (Fig. 2). Significantly, this binding was unaffected by human fibrinogen at 4 mg/ml when it was used as an inhibitor. In the same experiment a control antibody specific for digoxin (*12*) did not bind to the fibrin-coated disks.

Since the hybridomas were selected on the basis of fibrin monomer specificity, peptide inhibition of binding by the monoclonal antibodies was of interest. It appears that the synthetic β-fibrin peptide Gly-His-Arg-Pro-Leu-Asp-Lys-Cys inhibited each monoclonal antibody, whereas the control α-fibrin peptide inhibitor Gly-Pro-Arg-Val-Val-Glu-Arg-Cys did not (Table 1). These results show that the fibrin-specific monoclonal antibodies were elicited against the synthetic fibrin-like antigen and were not fortuitously selected by the hybridoma screening procedure.

The fibrin-disk assay was used to examine the species specificity of monoclonal antibody to fibrin 59D8. For each species tested, fibrin disks were prepared by means of the procedure described above, starting with fibrinogen from the cow, dog, chicken, pig, rabbit, and sheep (all obtained from Sigma, with the exception of chicken fibrinogen, which was prepared by ethanol precipitation). The direct binding of antibody 59D8 was measured (as described in the legend to Fig. 2) by using ^{125}I-labeled (5×10^4 count/min) goat antibodies to mouse Fab fragments. From the mean of two fibrin-coated disks, the binding relative to that of disks coated with human fibrin was calculated; antibody 59D8 cross-reacted strongly with canine (108 percent) and rabbit (75 percent) clotted fibrin, partially with bovine (25 percent), porcine (26 percent), and ovine (25 percent) fibrin, and not at all with chicken fibrin (1 percent).

To minimize cross-reactivity with spontaneously formed thrombi during experimentation in vivo, the chicken was selected for the development of an ex vivo circulatory model for the detection of human fibrin. An extracorporeal chamber was attached by cannulas to a carotid artery and the contralateral jugular vein. The chamber was maintained at 37°C. At the beginning of the experiment, 50 μg of monoclonal antibody was injected into the jugular vein through the efferent cannula. Blood was then permitted to circulate through the chamber for 15 minutes. The ex vivo circulation was momentarily interrupted to permit the insertion of two fibrin-coated disks, each rolled into an S-shaped configuration. After a predetermined time, circulation was again interrupted to remove the disks, which were immediately washed with an excess of PBSA containing Trasylol (5 KIU/ml). The bound monoclonal antibodies were measured by in vitro incubation of the fibrin-coated disks for 1 hour with ^{125}I-labeled goat antibodies to mouse Fab fragments.

Table 2. Detection of human fibrin: chicken ex vivo circulatory model. In experiment 1, two disks containing 30 to 35 μg of human fibrin were placed in a cylindrical plastic chamber and exposed to intravenously administered 64C5 (50 μg) for the indicated period of time before in vitro treatment with the labeled second antibody. In experiment 2 the human fibrin–coated disks were subjected to plasminolysis in the ex vivo chamber, then removed from the chamber, assayed in vitro for residual fibrin by using 64C5, and quantified with the labeled second antibody. Data are means ± standard deviations for two separate determinations.

Experiment	Paper disk	Circulation ex vivo (minutes)	Counts per minute*
1. Ex vivo detection of fibrin disks by intravenous antibody 64C5	Fibrin-coated	0	1,935 ± 250
	Fibrin-coated	10	13,116 ± 450
	Fibrin-coated	60	15,396 ± 600
	Uncoated	60	442 ± 60
2. Stability of fibrin toward ex vivo circulation: in vitro assessment with antibody 64C5	Fibrin-coated	0	19,138 ± 570
	Fibrin-coated	10	18,697 ± 600
	Fibrin-coated	30	19,512 ± 500
	Fibrin-coated	60	18,732 ± 500

*Mean radioactivity associated with ^{125}I-labeled antibody to mouse Fab, used to detect monoclonal antibody 64C5 bound to human fibrin–coated disks.

Binding of intravenously injected 64C5 or 59D8 monoclonal antibodies to the disks rapidly exceeded 50 percent within 10 minutes, relative to the maximum values for control disks, which were immersed into a solution of the same antibody in vitro. Disks exposed to circulating chicken blood for 1 hour did not differ from unexposed disks in their binding of antibody 64C5 or 59D8. Table 2 lists the data obtained for antibody 64C5. In control ex vivo experiments, it was demonstrated that (i) disks that did not contain human fibrin failed to bind antibody when placed in the ex vivo chamber; (ii) under the same circumstances, disks did not bind ^{125}I-labeled goat antibodies to mouse Fab immunoglobulin G when the monoclonal antibody was not injected; and (iii) disks coated with human fibrin could be exposed to circulating blood of anesthetized chickens for 2 hours without loss of antibody recognition.

Thus, monoclonal antibodies elicited by a synthetic peptide based on the human sequence are capable of binding to human fibrin monomer as well as fibrin polymer in the presence of fibrinogen, both in vitro and ex vivo. Whether these antibodies have applications in clinical diagnosis is a subject of considerable interest.

The immunochemical strategy used in this study may well permit differentiation between other proteins and their circulating precursors in physiological fluids or in vivo. For example, available antisera to renin are incapable of distinguishing between active renin and its circulating inactive form. Antibodies recognizing an epitope unique to renin relative to prorenin would be valuable in determining the concentration of each of these components. Similarly, an antibody directed at the amino terminus of Factor XIIIa might be useful for its detection and localization in the presence of Factor XIII, the circulating precursor.

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α-Difluoromethylornithine-Induced Polyamine Depletion of 9L Tumor Cells Modifies Drug-Induced DNA Cross-Link Formation

Abstract. *Depletion of intracellular levels of polyamines, which are believed to have a role in the intranuclear stabilization of DNA, alters the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea and* cis*-diamminedichloroplatinum II in 9L rat brain tumor cells. Alkaline elution techniques were used to show that polyamine depletion alters the number of DNA cross-links formed by these cytotoxic agents.*

The polyamines putrescine, spermidine, and spermine have various functions in cells, including an apparent role in the stabilization of DNA (*1*). Polyamines stabilize cell-free DNA, thus inhibiting enzymatic degradation (*2*) and denaturation by x-rays (*3*) and heat (*4*). α-Difluoromethylornithine (DFMO), an enzyme-activated, irreversible inhibitor of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway (*5*), depletes polyamine in 9L rat brain tumor cells (*6*). Polyamine depletion appears to cause an alteration in the conformation of 9L cell DNA (*7*). These effects of polyamines on the structural integrity of DNA, the probable target for the cytotoxic action of many anticancer drugs, suggest a possible role for polyamine depletion in cancer chemotherapy. We have shown that depletion of polyamines in 9L cells modifies the cytotoxic effects of several antineoplastic drugs both in vitro and in vivo (*8*). However, the mechanism by which polyamine depletion alters the reaction between active drug species and DNA has not been definitively established. We now report that polyamine depletion changes the number of cross-links formed by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and *cis*-diamminedichloroplatinum II (*cis*-platinum).

The cytotoxicity of chloroethylnitrosoureas is thought to be the result of a sequence of reactions in which an initial alkylation of DNA by a chloroalkyl carbonium ion intermediate is followed by nucleophilic displacement of a primary chlorine group to form DNA interstrand cross-links (*9*). We have shown in 9L cells that polyamine depletion caused by DFMO does not affect the cytotoxicity of nitrosoureas that do not cross-link DNA, does not change the number of DNA monoadducts in cells treated with ^{14}C-labeled 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU), and does not alter the activity of several enzymes that repair alkylation damage (*8*). However, DFMO-induced polyamine depletion increases the cytotoxicity of the chlorethylnitrosoureas BCNU, MeCCNU, and chlorozotocin (*8*), all of which cross-link DNA.

cis-Platinum is an anticancer agent that is thought to kill cells by forming DNA cross-links (*10*). However, in contrast to results obtained with chloroethylnitrosoureas, the cytotoxicity of *cis*-platinum was decreased by DFMO-induced polyamine depletion (*11*). The two chlorine leaving groups of *cis*-platinum are separated by 3.3 Å (*12*), a distance similar to the interplanar separation of DNA bases (3.4 Å) (*13*). A number of nucleophiles in native DNA are separated by the same distance (*14*). Presumably, after the initial formation of a chloroplatinate-base covalent bond in native DNA, other base nucleophiles displace the second chlorine and form either intra- or interstrand cross-links. An alteration in DNA structure caused by polyamine depletion may change the spatial configuration of these nucleophiles, which could make the cross-linking reaction with *cis*-platinum mechanistically unfavorable.

Our data for the experiments with chloroethylnitrosoureas and *cis*-platinum were obtained with a colony-forming efficiency (cell survival) assay, which reflects the combined effects of many factors that lead to cell death. With this method it was not possible to determine directly the mechanism by which polyamine depletion affected the cytotoxicity of chloroethylnitrosoureas and *cis*-platinum. The sister chromatid exchange assay, which measures effects on the chromosomal level, is an established method used to measure damage to DNA (*15*). Polyamine depletion increased BCNU-induced and decreased *cis*-platinum-induced sister chromatid exchanges, the same relative changes observed in the cytotoxicity experiments (*16*), which suggests that polyamine deficiency alters drug-induced DNA damage.

Our data suggested that the modification of cytotoxicity is the result of a change in the number of DNA cross-links formed by these anticancer drugs. We therefore used the alkaline elution assay to measure changes in the number of DNA cross-links formed by treatment with BCNU and *cis*-platinum in polyamine-depleted 9L cells. In the alkaline elution assay, cells are lysed on a filter, and the rate at which single-stranded DNA passes through the filter is determined (*17*). The presence of strand breaks increases the rate of elution. For the determination of cross-links, cells are irradiated before lysis to induce a fixed number of random single-strand breaks. Cross-linking between DNA strands or between DNA and protein is the source of the larger molecular species that remain after lysis. The presence of these larger species reduces the effects of irradiation and decreases the amount of DNA eluted from the filter. In the presence of proteinase K, the number of DNA-protein cross-links is greatly reduced; thus, if the enzyme is present in the lysing solution, the number of DNA interstrand cross-links is measured (*17*).

The 9L cells were seeded and cultured as described for our cytotoxicity and sister chromatid exchange experiments (*11, 16*). Cells were incubated with 1 m*M* DFMO for 72 hours and then treated with BCNU or *cis*-platinum for 1 hour. This procedure was also followed in experiments in which putrescine was added to the culture medium to replenish intracellular polyamine levels; after 48 hours of incubation with DFMO, putrescine was added to achieve a final concentration of 1 m*M*. After drug treatment, cells were incubated for 6 hours, the approximate length of time required for the maximum number of cross-links to form for 9L and other cell lines (*18*). The alkaline elution assay and the fluorometric quantitation of DNA were performed as described by Murray and Meyn (*19*).

Treatment of 9L cells with DFMO alone had no effect on the elution profiles