

Doxorubicin conjugates of monoclonal antibodies to hepatoma-associated antigens

(hepatocellular carcinoma/immunochemotherapy/immunoconjugates/hepatitis B virus surface antigen/ α -fetoprotein)

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Contributed by Michael Sela, July 28, 1988

ABSTRACT A panel of six murine monoclonal antibodies against hepatocellular carcinoma-associated antigens, reactive with PLC/PRF/5 human hepatoma cells, was conjugated to Adriamycin (doxorubicin) via a dextran bridge. This library of antibodies includes three monoclonal antibodies against hepatitis B virus surface antigen, one anti- α -fetoprotein, and two other IgG2a antibodies against PLC/PRF/5 hepatoma-associated antigens. The use of dextran for conjugation of Adriamycin to antibodies enabled a 5- to 10-fold amplification of the number of drug molecules linked to antibody. Conjugation of Adriamycin to dextran caused an occasional reduction in the pharmacologic activity of dextran-Adriamycin in [³H]thymidine incorporation assays in hepatoma cells as compared to nonconjugated Adriamycin. This loss of anticellular activity was partially compensated for by conjugation of specific antibodies to the dextran-Adriamycin conjugate. Conjugated compounds completely retained their binding activity to purified hepatitis B virus surface antigen and α -fetoprotein fixed to a solid matrix as compared to binding of homologous nonconjugated antibodies. However, some reduction of the binding activity to intact hepatoma cells was observed in three of six conjugates. Binding activity to hepatoma cells and, as a consequence, suppression of tumor cell DNA synthesis by the various conjugates was enhanced as compared to the same effect in treated colorectal carcinoma cells that do not express the relevant hepatoma-associated proteins. Furthermore, two conjugates containing nonspecific antibodies did not bind to hepatoma cells and caused minimal suppression of DNA synthesis. These results suggest that this panel of monoclonal antibody-dextran-Adriamycin conjugates was effective in suppression of PLC/PRF/5 cell growth *in vitro*.

Available chemotherapy for primary hepatocellular carcinoma (HCC) is not effective and usually produces only a partial remission. For example, Adriamycin (Adr; doxorubicin) is currently used for chemotherapy in HCC, leading to variable remission in 11–25% of patients (1). Its use, however, is restricted because of toxicity to normal tissues. Specific targeting of Adr to tumor cells may reduce its systemic toxicity and improve treatment efficacy. Conjugation of chemotherapeutic agents to carrier molecules such as polyclonal or monoclonal (mAbs) antibodies has been suggested (2, 3). We, and others, have previously reported the linking of such agents to antibodies either directly or via inert macromolecules through covalent binding of drug and antibody (3–6). In a previous study (7, 8), we have shown that daunomycin conjugates attached via a dextran (Dex) bridge to polyclonal antibody and mAb anti-rat α -fetoprotein (AFP) were cytotoxic to rat AH 66 hepatoma cells *in vitro* and *in*

vivo. The present report summarizes the results of *in vitro* experiments, utilizing a panel of murine mAbs against human hepatoma-associated antigens to which Adr was linked covalently via a Dex bridge.

MATERIALS AND METHODS

mAbs. The preparation of a panel of mAbs to hepatoma-associated antigens that are expressed by human hepatoma cell line PLC/PRF/5 has recently been reported (9–11). This library includes three anti-hepatitis B virus surface mAbs (anti-HBs) to hepatitis B virus surface antigens (HBsAg) of the IgG1, IgG2a, and IgM isotype, an IgG1 anti-AFP mAb to AFP (7); and two IgG2a mAbs to hepatoma-associated antigens designated anti-PLC₁ and anti-PLC₂. All antibodies bind specifically to PLC/PRF/5 cells, and antibodies anti-PLC₁ and anti-PLC₂ also bind to two other human hepatoma cells SK-Hep1 and Mahlavu. For control experiments, we used mAbs B₂TT₁ (IgG1 and IgG2a) directed against tetanus toxoid and mAb H-28 (IgM) against influenza virus hemagglutinin. Both antibodies do not bind to human hepatoma cells (9). Antibodies were partially purified from ascitic fluid of mice injected with somatic cell hybrid clones, using ammonium sulfate precipitation (IgG1), affinity purification with protein A-Sepharose 4B (IgG2a), and AcA 34 (IgM) columns as described (9).

Cell Lines. Human hepatoma cell lines PLC/PRF/5 (12), SK-Hep1 (13), and Mahlavu (14), as well as colorectal carcinoma cells SW948, used for control experiments, were grown in culture as monolayers with RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, nonessential amino acids, and antibiotics, and cells were harvested by trypsin/EDTA as described (15). PLC/PRF/5 and Mahlavu hepatoma cells contain integrated hepatitis B virus DNA, but only PLC/PRF/5 cells express and secrete HBsAg and AFP (15). The three hepatoma cell lines express two hepatoma-associated antigens—HAA₁ and HAA₂—of 70 and 50 kDa, respectively (10, 11).

Conjugation of Adr to mAbs. Purified mAbs were coupled to oxidized Dex as described (3). Briefly, 1 g of Dex T10 (10 kDa) (Pharmacia, Uppsala) was dissolved in 100 ml of 0.03 M NaIO₄ in 0.1 M sodium acetate (pH 5.5) and incubated for 20 hr at 4°C. Oxidized Dex was repeatedly dialyzed against distilled water and lyophilized until used. Dex (60 mg) in 2.5 ml of phosphate-buffered saline was added to a solution of Adr (18 mg) (a generous gift from Farmitalia, Carlo Erba, Milan) in 1 ml of water and incubated for 48 hr at 4°C. Finally, 1 ml of 12–15 mg of purified mAb was added and incubation

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Abbreviations: mAb, monoclonal antibody; HBsAg, hepatitis B virus surface antigen; anti-HBs, mAbs to HBsAg; AFP, α -fetoprotein; Adr, Adriamycin (doxorubicin); Dex, dextran; HCC, hepatocellular carcinoma.

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continued for 48 hr at 4°C. The conjugate mAb-Dex-Adr was then purified by separation on a Sephadex G150 or Bio-Gel P60 (Bio-Rad) column. The final preparations were passed through a 0.22- μ m filter. Conjugation of Adr to mAb was performed at least three times for each antibody and 37 batches were prepared. All conjugates were tested for their binding activity to intact hepatoma cells. Those conjugates containing mAb anti-HBs and anti-AFP were also tested for their binding activity to purified HBsAg and AFP fixed to a solid matrix. Only conjugates that retained 60% or more of their binding activity as compared to nonconjugated homologous mAb were then evaluated in a [³H]thymidine incorporation assay. All experiments were repeated three times or more for each conjugate.

Determination of Antibody Binding Activity of the Conjugate. mAb binding activity of the various conjugates or their individual uncoupled antibodies to the target proteins was tested by a variety of direct and indirect assays as described (3, 9–11, 16). Binding activity of the conjugates was also compared to binding of the uncoupled homologous mAb to PLC/PRF/5 cells. Specificity of binding was determined for each antibody by comparing the binding of ¹²⁵I-labeled mAb to its target before and after addition of nonlabeled homologous antibody or conjugate at a 10 to 100-fold excess (10). Finally, once direct binding activity and specificity for each conjugate were determined, a comparative indirect binding assay was performed simultaneously for all six conjugates and controls (11).

Assessment of Conjugate Pharmacologic Activity. Cytotoxic effects of the mAb-Dex-Adr conjugates and their individual components against hepatoma cells and control colorectal carcinoma cells were evaluated by a 4-hr [³H]thymidine incorporation assay (short assay). In some experiments, target cells were incubated with conjugates and [³H]thymidine for 16 hr (extended assay). Cells were then harvested by 0.1 M NaOH treatment (Titertek cell harvester Flow Laboratories) and counted in a γ -counter. The uptake of [³H]thymidine by target cells in the presence of conjugates and its components was expressed as percentage incorporation compared to a control assay in which tumor cells were incubated with complete medium. In some experiments (see Table 3 and Fig. 3), inhibition of thymidine incorporation was calculated according to the following formula: $A = (B - C)/(B \times 100)$, where A represents net percentage inhibition of thymidine incorporation by conjugate, B is the incorporation of thymidine in the presence of nonconjugated homologous mAb (dpm), and C is incorporation in the presence of conjugate (dpm).

Data were expressed as mean of quadruplicate determinations for binding and thymidine incorporation assays \pm SEM. Significance was evaluated by Student's t test.

RESULTS

Binding Activity of Conjugates to Their Target. Conjugates mAb-Dex-Adr containing antibodies anti-HBs (16) and anti-AFP completely retained their binding activity to purified HBsAg as compared to nonconjugated homologous mAbs. All six conjugates bound specifically to PLC/PRF/5 hepatoma cells but not to human colorectal cells using either a direct binding assay with ¹²⁵I-labeled conjugates, or in experiments in which nonlabeled conjugates caused a dose-dependent inhibition of binding of the homologous ¹²⁵I-labeled mAb to hepatoma cells (data not shown). The binding activities of the different conjugates to hepatoma cells were compared in an indirect assay performed simultaneously for the entire panel. As shown in Fig. 1, conjugates containing mAbs anti-HBs IgG2a and IgM as well as anti-AFP completely retained their binding activity to hepatoma cells (Fig. 1 B–D). Some loss of binding activity was observed for

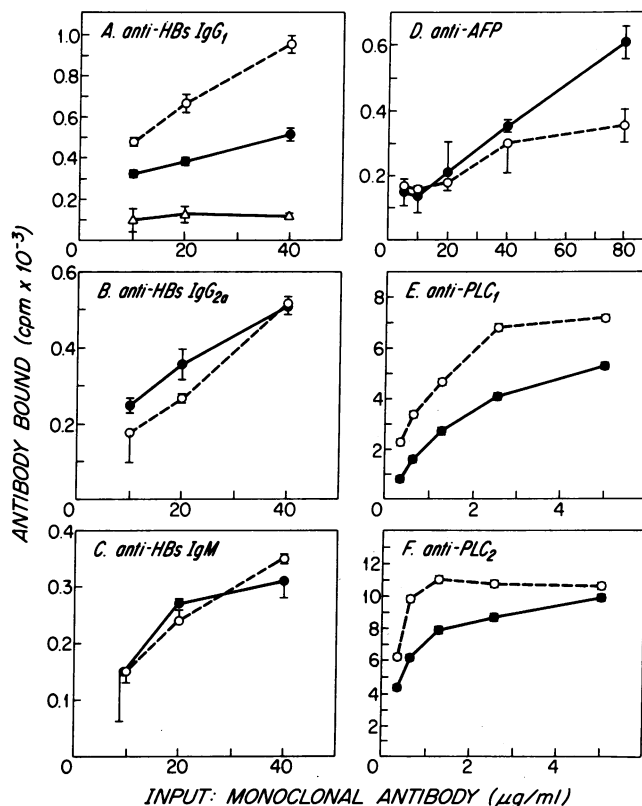


Fig. 1. Binding of conjugates to PLC/PRF/5 hepatoma cells. Cells (10^5 cells per well) were incubated in filter-covered microtiter plates for 1 hr at 24°C with conjugates mAb-Dex-Adr (●); a homologous nonconjugated mAb (○); a nonrelevant control nonconjugated IgG mAb B₂TT₁ (△). Cells were washed and ¹²⁵I-labeled goat anti-mouse F(ab')₂ was added (10^5 cpm per well) for 1 hr. Filters were washed and counted. Binding activity is expressed as mean cpm of quadruplicate determinations \pm SEM. Adr concentration (μ g/ml) in conjugates at 10 μ g of mAb per ml was for A, 1.50; B, 0.50; C, 1.25; D, 0.63; E, 0.50; F, 0.84. Binding of a Dex-Adr conjugate was 112 ± 2 dpm at an Adr concentration of 2 μ g/ml.

conjugates containing mAbs anti-HBs (IgG₁), anti-PLC₁, and anti-PLC₂ as compared to binding of the nonconjugated homologous mAbs to the target cells (Fig. 1 A, E, and F).

Effect of Conjugates on [³H]Thymidine Incorporation into Hepatoma Cells. In preliminary experiments, nonconjugated antibodies caused a variable enhancement of [³H]thymidine incorporation into target cells. This stimulatory effect was usually dose dependent and was caused by most mAbs tested, including nonrelevant control mAbs. However, the degree of stimulation varied between mAbs tested and was maximal for anti-AFP (70% enhancement over baseline values at 0.5 μ g/ml) and minimal for the nonrelevant control mAb against influenza virus hemagglutinin (9% enhancement at 0.5 μ g/ml).

Incubation of PLC/PRF/5 cells with conjugates containing specific mAbs IgM or IgG caused significant inhibition of [³H]thymidine incorporation into PLC/PRF/5 cells (Figs. 2 and 3; Tables 1 and 2). Conjugation of Adr to Dex led to a reduction of the pharmacological activity of Adr. This was observed in short incubation assays in which the free drug seems to penetrate the target cells rapidly. However, this difference was not observed in experiments in which incubation with Dex-Adr or the complete conjugate was extended to 16 hr (Fig. 2). Furthermore, the loss of activity of Adr after conjugation to Dex was partially compensated for after conjugation of Dex-Adr to specific mAbs as shown in Figs. 2 and 3. The relative specificity of treatment was demonstrated for the antibody as well as for the target used.

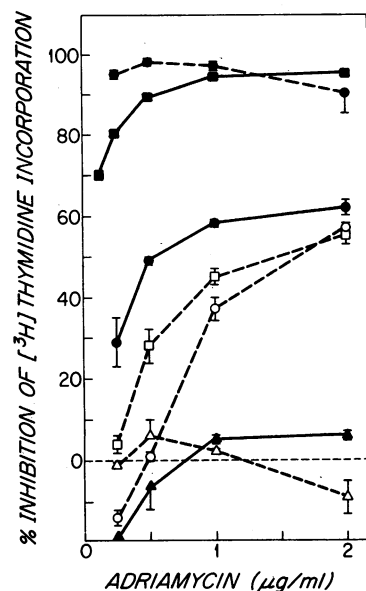


FIG. 2. Pharmacologic effect of conjugates. Inhibition of thymidine incorporation into hepatoma cells. PLC/PRF/5 cells (10^4 cells per well) were incubated with the conjugate or its individual components for 4 hr (short assay), followed by washing and incubation with [3 H]thymidine ($1 \mu\text{Ci}$ per well; $1 \text{ Ci} = 37 \text{ GBq}$) for 16 hr. In extended assays, cells were incubated with conjugate and [3 H]thymidine for 16 hr. Then, 0.1 M NaOH -treated cells were harvested and counted. Data are expressed as mean cpm of quadruplicate determinations \pm SEM. Short assay, anti-HBs (IgM)-Dex-Adr (\bullet - \bullet), anti-HBs (IgM) (\blacktriangle - \blacktriangle), anti-influenza (IgM)-Dex-Adr (\circ - \circ), anti-influenza (IgM) (\triangle - \triangle), Dex-Adr (\square - \square), Adr (\blacksquare - \blacksquare). Extended assay, anti-HBs (IgM)-Dex-Adr (\bullet - \bullet). Points below the 0 line on the horizontal axis represent stimulation. At Adr concentration of $2 \mu\text{g/ml}$, mAb anti-HBs was $3.8 \mu\text{g/ml}$, and mAb anti-influenza was $1 \mu\text{g/ml}$.

Conjugates containing mAb anti-HBs, anti-PLC₁, anti-PLC₂, and anti-AFP were significantly more inhibitory as compared to conjugates containing nonrelevant control antibodies such as anti-influenza-Dex-Adr (Table 1; Figs. 2 and 3). Moreover, the effect of relevant antibodies containing conjugates was more pronounced against hepatoma cells as compared to the effect on colorectal carcinoma cells (Table 1). Finally, conjugates containing mAbs anti-PLC₁ and anti-PLC₂ were also effective against PLC/PRF/5, as well as against SK-Hep1 and Mahlavu hepatoma cells (Table 2). The pharmacologic activity *in vitro* of the conjugates may vary between batches using the same antibody as well as between conjugates containing different mAbs because of a varying degree of drug substitution (range, 20–50 mol of drug per mol of mAb). In Table 3, the relative efficacy of the individual conjugates is compared under partially defined conditions. These results indicate that specific conjugates were superior to nonspecific conjugates as well as to Dex-Adr in inhibition of thymidine incorporation into hepatoma cells. Finally, among this panel of six immunoconjugates tested *in vitro*, compounds containing anti-HBs mAbs of all three isotypes were the most potent inhibitors of cellular DNA synthesis, while the conjugate containing anti-AFP mAb was shown to be the least effective.

DISCUSSION

The generation of mAbs to tumor-associated antigens has enabled the development of a wide range of potential applications such as serologic and pathologic diagnosis, radioimmunolocalization, and immunotherapy (4–6, 17). We have recently established a panel of mAbs reactive with human

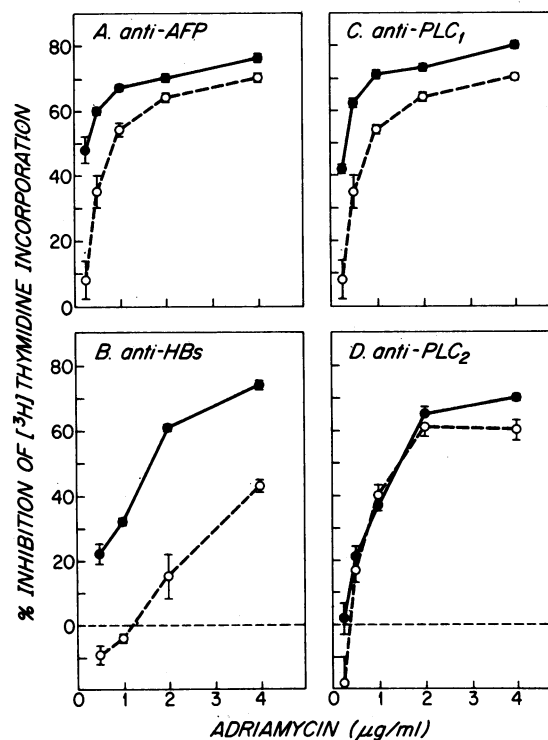


FIG. 3. Antibody-mediated enhancement of pharmacologic effect of conjugates. PLC/PRF/5 cells were incubated with conjugates (\bullet) or Dex-Adr (\circ) in a short [3 H]thymidine incorporation assay as described for Fig. 2. At an Adr concentration of $4 \mu\text{g/ml}$, mAb concentration was as follows: A, $12 \mu\text{g/ml}$; B, $1.9 \mu\text{g/ml}$ of anti-HBs (IgG2a) per ml; C, $7.5 \mu\text{g/ml}$; D, $1.2 \mu\text{g/ml}$. At the same Adr concentration, $P < 0.002$ for A, B, and C, and $P < 0.02$ for D.

hepatoma-associated antigens (9, 10). One of these antigens is membrane associated (HAA₂), while others are secreted in various amounts by different hepatoma cells (HBsAg, HAA₁, AFP). All these antigens are expressed by a human hepatoma cell line, PLC/PRF/5, and their respective antibodies bind specifically to these cells. We used this model system *in vitro* to assess the option of immunochemotherapy. In previous experiments, it was shown that treatment of PLC/PRF/5

Table 1. Suppression of [3 H]thymidine incorporation in hepatoma cells by anti-HBs-Dex-Adr

Treatment	[3 H]Thymidine incorporation, %	
	Hepatoma cells	Colorectal cells
Anti-HBs (IgG2a)	130 ± 2	NT
Anti-HBs (IgM)	118 ± 9	NT
Anti-influenza (IgM)	109 ± 2	NT
Adr	36 ± 2	76 ± 5
Dex-Adr	72 ± 5	82 ± 6
Anti-HBs (IgG2a)-Dex-Adr	78 ± 3	99 ± 7
Anti-HBs (IgM)-Dex-Adr	51 ± 2	124 ± 12
Anti-influenza (IgM)-Dex-Adr	96 ± 5	98 ± 7

PLC/PRF/5 hepatoma or SW948 colorectal cells were incubated with conjugates or their individual components for 4 hr at 37°C , washed, and incubated with [3 H]thymidine as described (10). Data are expressed as mean of quadruplicate determinations \pm SEM. In this experiment, baseline activity for [3 H]thymidine incorporation was established by incubation of target cells with complete medium in the absence of antibodies (100%). Data of $>100\%$ indicate stimulation and $<100\%$ indicate inhibition of thymidine incorporation. Adr concentration was $0.5 \mu\text{g/ml}$ and mAb was 0.25 , 0.5 , and $1.0 \mu\text{g/ml}$ for anti-influenza (IgM), anti-HBs (IgG2a), and anti-HBs (IgM), respectively. NT, not tested.

Table 2. Effect of conjugates on [³H]thymidine incorporation into hepatoma cells

Conjugate	[³ H]thymidine incorporation, % inhibition		
	PLC/PRF/5	SK-Hep1	Mahlavu
Anti-PLC ₁ -Dex-Adr	79 ± 5**	66 ± 4**	58 ± 4
Anti-PLC ₂ -Dex-Adr	65 ± 2	74 ± 1*	54 ± 1
Anti-AFP-Dex-Adr	71 ± 1**	NT	NT
Dex-Adr	61 ± 3	57 ± 1	52 ± 4

Conditions were as described in Fig. 2. Adr concentration was 2 µg/ml and mAb concentration was for anti-PLC₁, 1.25 µg/ml; anti-PLC₂, 0.6 µg/ml; anti-AFP, 6 µg/ml. NT, not tested. *P < 0.01; **P < 0.05 as compared to Dex-Adr.

tumor-injected athymic mice with nonconjugated monoclonal anti-HBs (IgG2a or IgM) provided a protective effect against tumor formation. However, in some instances, treatment had led to "escape" tumors that were 3-fold larger than tumors in untreated animals (17-19). To prevent such an escape phenomenon, several options are now being explored as shown in the present investigation. These include generation of additional mAbs to hepatoma-associated antigens, which may be administered sequentially or in combinations (10, 11) and the construction of conjugates between the above antibodies and Adr.

Adr is one of the few available chemotherapeutic agents for HCC, but its use is mainly restricted because of its cardiac and bone marrow toxicity. Treatment with mAbs conjugated with Adr should theoretically increase the drug concentration at the tumor site while reducing its systemic side effects. Conjugation of Adr to polyclonal antibodies or to mAbs via a Dex bridge has been reported (3) and Dex has been used in humans as an inert, soluble, and nontoxic volume expander. The use of Dex leads to a 10- to 50-fold amplification of the number of Adr molecules coupled to antibody. Although the controlled oxidation of Dex enables the conjugation of drug and antibody, batch to batch variation among conjugates may fluctuate between 10% and 30% in terms of drug substitution, even under optimal conditions. The conjugation process of Dex to drug or antibody depends on the available free aldehyde groups on the oxidized spacer. These aldehyde groups are then bound to functional free amino groups of drug and of antibody through the formation of Schiff bases. Three of the six conjugates reported in the present study maintained their binding activity to the target cells as compared to nonconjugated homologous mAbs, while in the remaining three conjugates a reduction in the binding activity was observed. Nevertheless, this loss in binding activity was not present for binding of anti-HBs-Dex-Adr to purified HBsAg

Table 3. Comparative effect of conjugates on thymidine incorporation into hepatoma cells

mAb in conjugate	Adr concentration in conjugates causing 50% suppression of thymidine incorporation		Potentiation factor
	mAb-Dex-Adr, µg/ml	Dex-Adr, µg/ml	
Anti-HBs (IgG1)	0.8	3.0	3.8
Anti-HBs (IgG2a)	0.1	0.4	3.1
Anti-HBs (IgM)	0.8	2.4	3.0
Anti-PLC ₁ (IgG2a)	0.4	0.9	2.3
Anti-PLC ₂ (IgG2a)	0.9	1.2	1.3
Anti-AFP (IgG1)	17.4	3.5	0.2
Anti-tetanus (IgG)	1.3	1.2	0.9
Anti-influenza (IgM)	2.7	2.4	0.9

The potentiation factor describes the Dex-Adr/mAb-Dex-Adr ratio.

(16) as opposed to binding of conjugate to PLC/PRF/5 hepatoma cells, thus reflecting a difference in the two assays. Furthermore, this loss of activity was partially compensated for by increasing the concentration of mAbs or prolonging the incubation time. It should, however, be noted that the relative binding activity of each of the conjugates to the target cells and probably also their pharmacologic activity may be partially dependent on the number of binding sites present on the target cell surface. The number of binding sites for each mAb used varies and is higher in PLC/PRF/5 cells for mAb anti-PLC₁ and anti-PLC₂ than for anti-HBs or anti-AFP. This difference is also reflected in the minimal concentration of Adr in the various conjugates, which is effective in inhibition of [³H]thymidine incorporation.

Conjugation of Adr to Dex led in some experiments to a 30-50% reduction in drug activity as measured by a short [³H]thymidine incorporation assay. This loss of pharmacologic activity of Dex-Adr as compared to free Adr was partially compensated for by conjugation of the specific antibodies. Full compensation was achieved when incubation time between conjugate and target cells was extended from 4 to 16 hr. Despite the loss in binding activity after the conjugation process, conjugates containing specific mAbs were always significantly more potent as compared to Dex-Adr or to conjugates containing nonspecific mAbs that do not bind to the target cells. Such a synergistic effect between mAb and Dex-Adr was previously observed in experiments in which anti-HBs Dex-Adr was more cytotoxic to hepatoma cells as compared to the effect of free Adr in a ⁵¹Cr release assay (16). The strategy of using conjugates may have some more limitations in view of the fact that some nonconjugated mAbs caused stimulation of thymidine incorporation in hepatoma cells. Furthermore, PLC/PRF/5 hepatoma cells secrete HBsAg and AFP, which could interfere with the binding of their specific mAbs to the target by the formation of immune complexes. It was therefore surprising to observe that conjugates containing monoclonal anti-HBs, regardless of isotype used, were the most potent inhibitors of DNA synthesis among the conjugates tested. These results support previous observations in which treatment of athymic mice with nonconjugated mAb anti-HBs (IgG2a and IgM) lead to an impressive protection against s.c. PLC/PRF/5 tumor growth in 75% of treated athymic mice, despite the presence of circulating HBsAg (17, 18). In contrast, conjugates containing mAb anti-AFP were the least effective in the present study, despite their preserved binding activity to the target cells, as reflected by the relatively large concentration of Adr necessary to produce a 50% inhibition of thymidine incorporation *in vitro*.

The mechanism(s) by which conjugates cause injury to hepatoma cells has not been established yet. In preliminary experiments, there is some evidence that conjugates may react with the hepatoma cell membrane in a manner independent from the complement-mediated injury reported previously for mAbs anti-HBs IgG2a and IgM (9). It is unknown whether conjugates are internalized intact or disperse into their individual components upon contact with the cell surface, and further studies are required.

In summary, we have shown that conjugates between mAbs to hepatoma-associated antigens and Adr are highly effective *in vitro* in causing specific target cell injury. The use of this strategy may permit more effective tumor cell lysis and reduce systemic toxicity of Adr.

This study was supported in part by the United States-Israel Binational Science Foundation, Grant 3158/84 and by the Richard Molin Memorial Foundation for Cancer Research and Grants CA-35711, AA-02666, and HD-20469 from the National Institutes of Health. J.R.W. is the recipient of Research Career Scientist Development Award AA-00048. We are grateful to Rolf Carlson for his excellent technical assistance.

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