MONOCLONAL ANTIBODY RAISED AGAINST APOPROTEIN OF C-1027: EFFECT ON BIOCHEMICAL AND BIOLOGICAL ACTIVITIES OF THE HOLOANTIBIOTIC

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A monoclonal antibody raised against C-1027 apoprotein recognized not only the apoprotein, but also the holoantibiotic (Antibiotic C-1027) almost equally. Among the biochemical and biological activities of the holoantibiotic, the antibody inhibited the aminopeptidase activity and the cytotoxicity to Ehrlich carcinoma cells in cultures, but not the DNA-cleaving activity *in vitro*. The immunohistogram, using this antibody, of Ehrlich carcinoma cells that had been exposed to the holoantibiotic suggested penetration of the holoantibiotic into target cells.

Antitumor antibiotic C-1027 consists of an enediyne chromophore[1] and a non-covalently bound apoprotein with 110 amino acid residues[2,3]. Like other members of the chromoprotein family, the chromophore causes strand scission of DNA *in vitro* and *in vivo* and is responsible for the cytotoxicity of the holoantibiotic, while the apoprotein stabilizes the chromophore[4]. In a previous paper, we presented data suggesting that the apoprotein functioned not only as a simple stabilizer but also as a targeting vehicle for the chromophore[5]. To confirm the role of the apoprotein in the action of the holoantibiotic, we studied the effect of a monoclonal antibody raised against the apoprotein (free of chromophore) on several indices of the holoantibiotic's activity. The present paper describes these results.

Methods

Anti-apoprotein IgG and its Specificity

As an antigen to produce a hybridoma cell line, the apoprotein but not the holoantibiotic was used because of the strong cytotoxicity of the latter. Selection of the hybridoma cell line and affinity purification of the monoclonal antibody were conducted at Biotechnology Research Laboratories, Takara Shuzo Co., Ltd., Japan. Immunochemical specificity of this antibody was determined as follows. The apoprotein or the holoantibiotic was immobilized in wells of microtiter plates using Takara Peptide Coating Kit (Takara Shuzo), according to the manufacture's instructions. In brief, 50 μl of either the apoprotein or the holoantibiotic (4 μg/ml of Reaction Buffer) and 10 μl of Coupling Reagent were added successively to each well and the plates were kept at room temperature for 2 hours. The wells were washed with distilled water, laid upside-down on a sheet of filter paper to remove water, treated with 200 μl/well of Blocking Reagent for an hour at room temperature, washed with PBS three times, incubated with 100 μl/well of Anti-apoprotein IgG (0.5~10 μg/ml) for an hour at 37°C in the dark, and washed with PBS. The wells were incubated with 100 μl/well of horseradish peroxidase-conjugated goat antibody to mouse IgG (Cappel #55563, affinity purified and 1/500 diluted with PBS-1% w/v BSA) at 37°C for an hour, washed with PBS, and incubated with 100 μl/well of 2,2'-azino-bis-(3-ethylbenzthiazoline 6-sulfonate) (Boehringer Mannheim Biochemica, Catalog No. 1112422, 1 mg/ml in 1 μM Citrate Buffer, pH 5, -0.01% H_2O_2) at

37°C for 30 minutes. Reaction (peroxidase activity) in each well was read at 405 nm on a microplate reader.

Effect of Anti-apoprotein IgG on the Cytotoxicity of the Holoantibiotic

Ehrlich carcinoma cells (ATCC CCL 77) were seeded at 10^4 cells/ml DMEM-5% CS/well (2 cm^2) in Coster 24-well tissue culture clusters and incubated at 37°C in 5% CO_2-humidified air (day 0). On day 1, the holoantibiotic or a mixture of the holoantibiotic and anti-apoprotein IgG was added to the wells. After incubation for 2 hours, the cells were washed free of the holoantibiotic and the IgG with 1 ml of PBS and incubated further in DMEM-5% CS until day 3. Cell growth was quantified by cell counting or by a colorimetric MTT assay[6] and the cell growth of a test run was expressed as a relative value calculated using the following equation:

$$\frac{\text{Cell number in a test run on day 3} - \text{Cell number on day 1}}{\text{Cell number in the control run on day 3} - \text{Cell number on day 1}} \times 100\,(\%)$$

Immunohistochemical Detection of the Holoantibiotic

Ehrlich carcinoma cells were suspended in DMEM-5% CS at 7×10^4 cells/ml and 0.15 ml portions therefrom were placed on cover glasses preset in 8 cm^2 dishes (Falcon 3001), and were incubated for 20 hours. The medium was removed, the cell layers were covered with 200 μl of a fresh medium containing 0.2 μg of the holoantibiotic and incubated for an hour. As a control, cells were incubated in a medium without the holoantibiotic. *In situ* staining of holoantibiotic (and possibly dissociated apoprotein also) was conducted by the streptavidin-biotin-peroxidase complex method (Histofine SAB Kit, Nichirei Japan) as follows. Cells adhering to each cover glass were freed of the medium by rinsing with 1 ml of cold PBS three times, and each cover glass was then immersed in 1 ml of acetone for 7 minutes at −20°C. The fixed cells were washed with 1 ml of cold PBS three times, blocked with 3% H_2O_2 for 15 minutes at room temperature, washed with 1.5 ml of cold PBS for 5 minutes three times, blocked with 10% normal goat serum for 15 minutes at room temperature and washed with cold PBS for 5 minutes three times. The cells were incubated with 100 μl of 70 μg/ml mouse anti-apoprotein IgG for an hour at 37°C, washed with cold PBS for 5 minutes three times, incubated with a mixture of biotinylated goat anti-mouse and -rabbit IgG for 10 minutes at room temperature and washed with cold PBS for 5 minutes three times. The cells were incubated with streptavidin-horseradish peroxidase complex for 5 minutes at room temperature, rinsed with cold PBS for 5 minutes three times, incubated with a mixture of 3,3'-diaminobenzidine·4 HCl, 0.2% H_2O_2 and buffer solution for 10 minutes at 37°C, rinsed with 2 ml of water 8 times, counterstained with MAYER's hematoxylin solution for 2 minutes at room temperature and washed with water 8 times. The stained cells were covered with 50% v/v glycerol/PBS (one drop on a cover glass) and inspected under a microscope.

Results and Discussion

The anti-apoprotein IgG equally recognized the apoprotein and the holoantibiotic, as Fig. 1 shows. Since there are some conformational differences between the apoprotein and the holoantibiotic[7], this antibody must recognize a local structure that was common to the apoprotein and the holoantibiotic.

Among the biological and biochemical activities of the holoantibiotics, the aminopeptidase activity only was found intrinsic to the holoantibiotic; neither the chromophore alone nor the apoprotein alone had this activity[5]. In contrast, the activity causing strand scission of DNA *in vitro* or in living

Fig. 1. Specificity of anti-apoprotein IgG.

○ 0.2 μg/well holoantibiotic, ● 0.2 μg/well apoprotein.

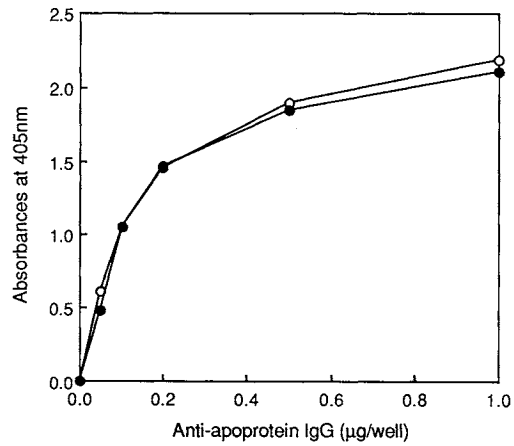


Table 1. Effect of anti-apoprotein IgG on the aminopeptidase activity of the holoantibiotic.

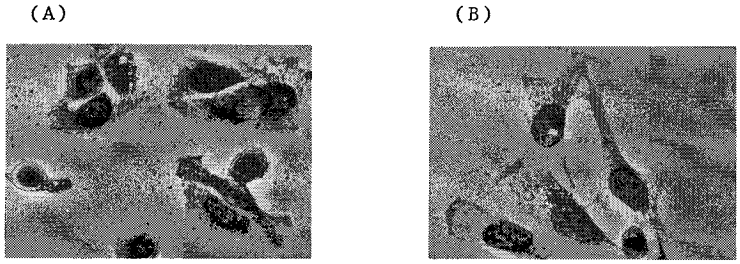
Substrate	Enzyme (holoantibiotic)	Antibody	Enzyme : Antibody (molar ratio)	Enzyme activity (%)
250 μM Phe-MCA	5 μg/ml	None		100
	5 μg/ml	50 μg/ml Anti-apoprotein IgG	1 : 0.75	21
	5 μg/ml	50 μg/ml Anti-Rat IgG[a]	1 : 0.75	111
250 μM Leu-MCA	5 μg/ml	None		100
	5 μg/ml	50 μg/ml Anti-apoprotein IgG	1 : 0.75	42

Molar ratios of enzyme vs antibody were calculated on the basis of the molecular weights of the enzyme (11.3 Kd) and the antibody (150 Kd). Aminopeptidase activity was determined as reported[5] by use of L-phenylalanyl 4-methyl-coumaryl-7 amide (Phe-MCA) or L-leucyl 4-methyl-coumaryl-7 amide (Leu-MCA) as the substrate.

[a] A negative control.

Fig. 2. Immunocytochemical detection of holoantibiotic in Ehrlich carcinoma cells.

Cells were incubated for 1 hour in the presence (A) or absence (B) of 1 μg/ml holoantibiotic and were stained by the immunochemical method as described under methods.



cells, antibacterial activity, the cytotoxicity to mammalian cells, *etc.*, are the characteristics of the chromophore, and hence, shown by the holoantibiotic as well as by the chromophore alone, though the latter is generally less active because of the instability. The apoprotein alone shows none of these activities. However, we previously presented data suggesting a role of the apoprotein as a targeting vehicle for the chromophore by binding to the cell surface structure that could be a substrate for the holoantibiotic as an aminopeptidase. We therefore determined possible effect of anti-apoprotein IgG on the aminopeptidase activity and on the cytotoxicity of the holoantibiotic. As Table 1 shows, the antibody strongly inhibited the enzyme activity, with Phe-MCA or Leu-MCA as the substrate. The cytotoxicity of the holoantibiotic to Ehrlich carcinoma cells was also lowered by the antibody in a dose-dependent manner, as Table 2 shows. These results, together with our previous finding that aminopeptidase inhibitors lower the cytotoxicity of the holoantibiotic[5], support the role of the apoprotein as a targeting vehicle. It is not known whether the apoprotein releases the chromophore at the cell surface or penetrates into cells accompanying the chromophore, *i.e.*, as the holoantibiotic. Attempts were made to trace the holoantibiotic in the cells that had been exposed to the antibiotic using the staining with the antibody. As Fig. 2 shows, the cells exposed to the holoantibiotic

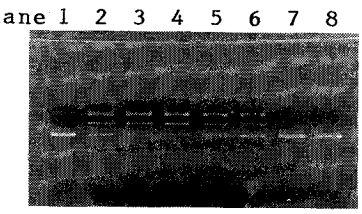
Table 2. Effect of anti-apoprotein IgG on the growth-inhibitory activity of the holoantibiotic against Ehrlich carcinoma cells.

Holoantibiotic (ng/ml)	Anti-apoprotein IgG (ng/ml)	Antibiotic : IgG (molar ratio)[a]	Growth (%)
None	None		100
3	None		23
3	15	1 : 0.4	31
3	60	1 : 1.5	46
3	245	1 : 6.2	74
None	245		102

[a] See the legend to Table 1.

were significantly stained, compared with the control, suggesting the penetration of the protein component, either as the holoantibiotic or as the dissociated apoprotein. Another chromoprotein antibiotic, neocarzinostatin, is also reported[8] to be taken up as a whole into cells, consistent with our observation. Considering the extremely unstable nature of the isolated chromophore, it seems likely that the chromophore is escorted by the apoprotein all the way through the cytoplasm and the nucleus until reacting with DNA. With regard to this possibility, it was an interesting finding that the IgG, even in a large excess, did not inhibit but rather stimulated DNA-cleaving activity of the holoantibiotic *in vitro*, as shown in Fig. 3. From X-ray crystallography of macromomycin, another member of the chromoprotein family, ROEY and BEERMAN proposed a three-dimensional structure of its apoprotein having a pocket to accomodate its chromophore[9]. A similar study is in progress with C-1027[10]. We presume that our IgG binds to the apoprotein at a locus remote from such a pocket structure, allowing the chromophore to retain the DNA cleaving activity.

Fig. 3. DNA strand scission by holoantibiotic in the presence or absence of anti-apoprotein IgG.



Strand scission of DNA by holoantibiotic *in vitro* were analyzed as reported[5] except that holoantibiotic and anti-apoprotein IgG were mixed and kept for 10 minutes at 0°C before the DNA strand scission assay. pBR322 DNA was incubated with none (lane 1), 2 μg/ml holoantibiotic (lane 2), 2 μg/ml holoantibiotic and 10 μg/ml anti-apoprotein IgG (molar ratio; 1 : 0.38, lane 3), 2 μg/ml holoantibiotic and 20 μg/ml anti-apoprotein IgG (molar ratio; 1 : 0.75, lane 4), 2 μg/ml holoantibiotic and 10 μg/ml anti-Rat IgG (molar ratio; 1 : 0.38, lane 5), 2 μg/ml holoantibiotic and 20 μg/ml anti-Rat IgG (molar ratio; 1 : 0.75, lane 6), 20 μg/ml anti-apoprotein IgG (lane 7) or 20 μg/ml anti-Rat IgG (lane 8).

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