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Target Specificity of ^{188}Re-labeled B27.1 Monoclonal Antibodies to Ovarian Cancer Cells *In Vivo*

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Abstract. *Background: Currently we are exploring a new multistep pretargeting approach involving administration of a bispecific antibody (B27.1 x P54) which has an anti-CA-125 (B27.1) and antibiotin (P54) paratope. It is followed by the administration of radiolabeled biotinylated liposomes to target the ^{188}Re to the ovarian cancer cells. As a preliminary step to realize this goal, we determined the target specificity of the monoclonal antibodies (B27.1) to the ovarian cancer cells in vivo. Materials and Methods: B27.1 monoclonal antibodies were photoreduced using UV light and incubated with reduced ^{188}Re for 30 min at 25°C. ^{188}Re-labeled B27.1 antibodies were purified using size exclusion chromatography. A comparative biodistribution of Re-B27.1 and ^{188}Re was performed in nude mice xenografted with NIH:OVCAR-3 cells. Results: While free rhenium distributed preferentially into thyroid and stomach with insignificant accumulation in the cancer cells, about 20% of the injected dose of ^{188}Re-B27.1 was recovered in ascites cells with insignificant localization in other organs four hours after administration. Conclusion: The study validates the affinity of the B27.1 antibodies to the ovarian cancer cells in vivo.*

Ovarian cancer ranks fifth in cancer deaths among women, accounting for more deaths than any other cancer of the female reproductive system. It was estimated that there will be about 15,280 deaths from ovarian cancer in the United States during 2007 (1). Ovarian cancer is considered to be a very chemosensitive malignancy (2, 3) and even after clinically and surgically defined complete response to the chemotherapy, majority of the women experience recurrence in two years. These later tumors generally do not respond to the chemotherapy due to the development of drug resistance and the tumor spreads into the peritoneal cavity resulting in development of ascites. Therefore, it was suggested that delivery of chemotherapy beyond the 'standard dosage regimen' (standard six courses) might be capable of killing the sensitive cells that were less vulnerable to cytotoxic agents when the primary treatment was administered (4). In addition, recent clinical studies have suggested that intraperitoneal therapy could be a more rational approach because ovarian cancer metastases are predominantly located within the peritoneal cavity (5). However, the intraperitoneal chemotherapy or radiation therapy cannot eradicate the microtumors (because they are difficult to access) and ultimately the 5 year survival rate of these advanced stage ovarian cancer patients is only 45% .

Monoclonal antibodies (MAbs) have been used as delivery vehicles for drugs, toxins and radionuclides. Many research groups have demonstrated tumor regression in experimental animals using radiolabeled monoclonal antibodies (6-9) and numerous investigators have tried the strategy in patients (10-14). Monoclonal antibodies developed against specific tumor antigens are rarely strongly cytotoxic and are therefore usually ineffective in tumor destruction. Though tumor cells expressing the specific antigen may some times undergo cell death, cells negative for this antigen will be spared. To circumvent these problems radionuclides may be attached to the antibody, which has the ability to kill both the antigen-positive and antigen-negative tumor cells in the vicinity of the former, via the bystander effect (15). However, one of the shortcomings of the application of directly radiolabeled MAbs is that only limited number of radioisotope molecules (between one-to-two) can

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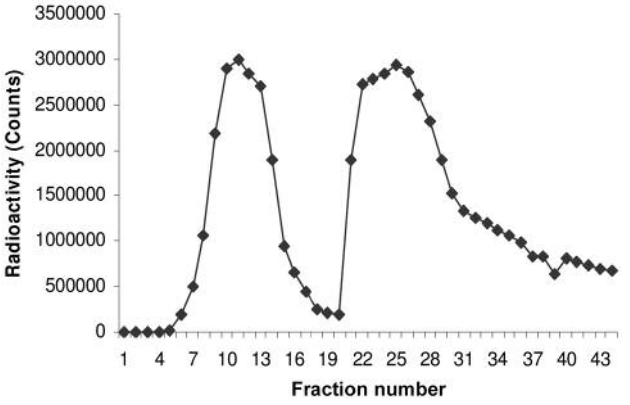

Figure 1. *Purification of ^{188}Re- labeled B27.1 antibody using Sephadex-G25 column chromatography. A known amount of antibody solution was incubated with reduced ^{188}Re for 30 minutes at 25˚C. The mixture was purified for rhenium labeled antibody by passing it through a Sephadex G-25 column and eluting with PBS pH 7.4.*

be conjugated per antibody necessitating high dose of the required antibody resulting in toxicity. This lead to the development of multistep pretargeting strategies to increase the tumor-to-normal tissue ratios of radioactivity and ultimately higher therapeutic efficacy (16).

Several pretargeting strategies have already been reported in which an antibody that is not rapidly internalized by the target cell is injected prior to the administration of radiolabeled molecule (or carrier particle) that has a strong affinity to the antibody (16, 17). Most of these strategies are based on high affinity of biotin-avidin binding, and investigators have used biotinylated MAbs to link to the radiolabeled biotin/streptavidin derivatives (16-19). Nevertheless, immunogenicity of avidin or streptavidin molecule might be a problem with repeated application to patients (20). In a study by Nassander *et al.*, (21), preferential uptake of doxorubicin loaded immunoliposomes was demonstrated, but because of premature release of the drug, no therapeutic advantage was obtained (22).

Currently we are exploring a new multistep pretargeting approach using the combination of a bispecific antibody (BsMAb) which has an anti-CA-125 and antibiotin paratopes, and biotinylated liposomes loaded with a cytotoxic radionuclide, ^{188}Re. It is known that 80% of the late stage ovarian cancer patients show high levels of tumor associated antigen, CA 125 (23, 24). Briefly, our strategy involves administration of the bispecific antibodies first. The bispecific antibody binds to the tumor cell surface. This step is followed by the administration of radiolabeled biotinylated liposomes to target the ^{188}Re to the cancer cells. ^{188}Re emits a high proportion of β (2.1 Mev) and a low proportion of γ-radiation (155 KeV) with penetration capacity of 11 mm and half-life of about 17 hours. These physical properties make ^{188}Re suitable for calibration and for radiotherapy (25, 26). We hypothesize that specific delivery of high concentrations of the ^{188}Re to the tumor cells can result in optimal killing of the less sensitive tumor cells with minimal systemic side effects. As a first step to realize this goal, B27.1 monoclonal antibodies were radiolabeled with ^{188}Re and their specificity to ovarian cancer cells was assessed in Balb/c nude mice xenografted with NIH OVCAR-3 cells.

Materials and Methods

Cells and cell culture. Human ovarian cancer cells NIH:OVCAR-3 were purchased from American Type of Culture Collection (ATCC, Rockville, MD, USA) and maintained at 37˚C and 5% CO_2 in RPMI-1640 medium (GIBCO, Grand Island, NY, USA). All experiments were performed with the cells in exponential growth phase.

Monoclonal antibodies. MAb B27.1 is a murine IgG1 with κ light chains, produced by immunizing mice with a partially purified CA-125 antigen (27). B27.1 antibodies recognize the glycoprotein CA-125 antigen on OVCAR-3 cell surface and have been used in radioimmunoassay, radioimmunodiagnosis and immunotherapeutic studies (27-29). Hybridoma secreting the MAbs were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The B27.1 hybridoma was kindly supplied by Biomira, Edmonton, Canada. B27.1 MAbs were purified from the hybridoma cell supernatants by Protein G affinity chromatography (Sigma, St. Louis, MO, USA).

^{188}Re reduction. ^{188}Re is an attractive therapeutic radioisotope which is produced from decay of the reactor-produced tungsten-188 parent ($t_{1/2}$ 69 days) and thus conveniently obtained on demand by elution from the alumina-based tungsten-188/rhenium-188 generator system. Rhenium-188 generator was obtained from Oakridge National Laboratories, Oak Ridge, TN, LI, USA. The rhenium-188 was obtained as sodium perrhenate by elution of the generator with 0.9% saline. ^{188}Re must be in a reduced state in order to label the antibody. Tin dihydrochloride (10 mg) was suspended in about 200 μL of water by sonication. A known volume of ^{188}Re stock solution was taken in a glass reaction vial and an equal volume of 0.1 M citrate buffer was added (pH 2-7). The tin dihydrochloride suspension was added to the above solution and allowed to react at 80˚C under nitrogen. After one hour, rhenium was tested for reduction using ITLC with acetone as the mobile phase. Under these conditions, perrhenate moved with the solvent front and reduced rhenium remained at the origin.

Photoactivation of the antibody. The hybridoma was grown for one month in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The culture supernatant was purified through Protein G affinity column chromatography to obtain B27.1 antibodies. A highly efficient photoreduction method was adopted for reducing the disulfide linkage of the antibody (30, 31). The photoactivation unit consists of a cylinder equipped with eight replaceable UV lamps of 300 nm (3.9W each), 254 nm (8.0 W each) and 365 nm (4.5W each) wavelength emission installed in a symmetrical manner. Cooling was effected by a fan at the bottom of the chamber. The antibody sample was introduced in a Quartz vial and placed at the base of the cylinder. The sample after exposure to UV light approximately for 60 minutes, was used for labelling experiments or stored at –20ºC until further use.

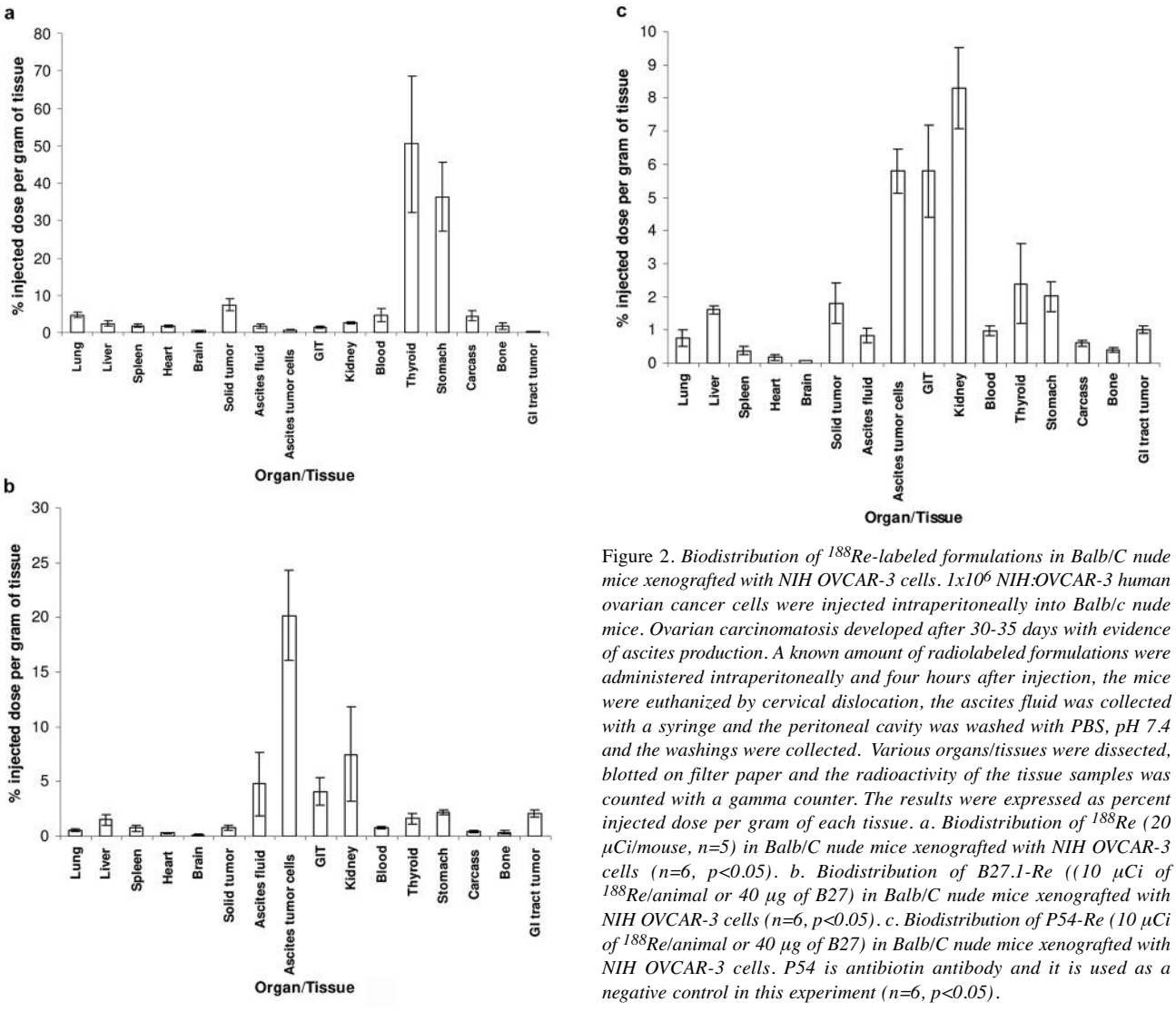

Figure 2. *Biodistribution of ^{188}Re-labeled formulations in Balb/C nude mice xenografted with NIH OVCAR-3 cells. 1x10^6 NIH:OVCAR-3 human ovarian cancer cells were injected intraperitoneally into Balb/c nude mice. Ovarian carcinomatosis developed after 30-35 days with evidence of ascites production. A known amount of radiolabeled formulations were administered intraperitoneally and four hours after injection, the mice were euthanized by cervical dislocation, the ascites fluid was collected with a syringe and the peritoneal cavity was washed with PBS, pH 7.4 and the washings were collected. Various organs/tissues were dissected, blotted on filter paper and the radioactivity of the tissue samples was counted with a gamma counter. The results were expressed as percent injected dose per gram of each tissue. a. Biodistribution of ^{188}Re (20 μCi/mouse, n=5) in Balb/C nude mice xenografted with NIH OVCAR-3 cells (n=6, p<0.05). b. Biodistribution of B27.1-Re ((10 μCi of ^{188}Re/animal or 40 μg of B27) in Balb/C nude mice xenografted with NIH OVCAR-3 cells (n=6, p<0.05). c. Biodistribution of P54-Re (10 μCi of ^{188}Re/animal or 40 μg of B27) in Balb/C nude mice xenografted with NIH OVCAR-3 cells. P54 is antibiotin antibody and it is used as a negative control in this experiment (n=6, p<0.05).*

^{188}Re labelling of the B27.1 antibody. After the antibody was photoreduced, a known amount of antibody solution was incubated with rhenium for 30 minutes at 25˚C. The mixture was purified for rhenium-labeled antibody by passing it through a Sephadex G-25 column and eluting with PBS pH 7.4. The photoreduced antibody was tested for immunoreactivity (antigen binding) using enzyme linked immunosorbent assay (ELISA).

In vitro stability studies. The stability of radiopharmaceuticals is usually analyzed by using different challenging agents, such as cysteine, human serum albumin *etc*. (32). In this study, an aliquot of ^{188}Re-labeled antibody solution was added to an equal volume of PBS pH 7.4 and incubated at 37˚C. An aliquot of the mixture was taken at different time intervals up to 12 hrs and the amount of free ^{188}Re in the sample was determined by Sephadex G25 column chromatography (Amersham Biosciences, NJ, USA). In another experiment, cysteine challenge was carried out by incubating an aliquot of the labeled antibody solution with equal volume of excess cysteine solution (50 mM) at 37˚C. Rest of the procedure was same as the above.

Biodistribution studies. 1x10^6 NIH:OVCAR-3 human ovarian cancer cells were injected intraperitoneally into Balb/c nude mice. Ovarian carcinomatosis developed after 30-35 days with evidence of ascites production and the occasional solid tumor attached to various peritoneal tissues. These tumor-bearing mice were used for biodistribution studies (33). The mice were divided into 3 groups of each six. To the first group, 0.5 mL of ^{188}Re solution (20 μCi/animal) was administered (*i.p.*). To the remaining two groups, Re-labeled B-27.1 solution (10 μCi of ^{188}Re/animal or 40 μg of B27.1) and the Re-labeled P54 (P54 is antibiotin antibody) were administered (*i.p.*) at the same antibody concentration separately. P54 antibody was used as a negative control in the experiment. Four hours after injection of each formulation, the mice were euthanized by cervical dislocation, the

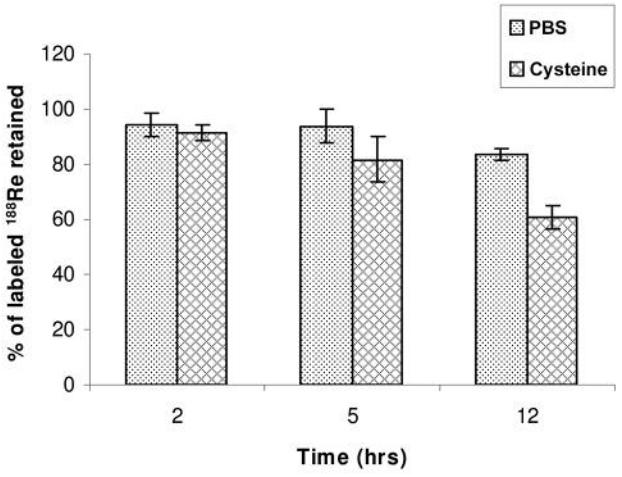

Figure 3. *Stability of ^{188}Re-labeled B27.1 antibodies. A known amount of ^{188}Re-labeled antibody solution was added to an equal volume of PBS pH 7.4, and equal volume of excess cysteine solution (50 mM) separately and incubated at 37°C. An aliquot of the mixture was taken at different time intervals, 2, 5, 12 hrs and the amount of free ^{188}Re in the sample was determined by Sephadex G25 column chromatography. Results are expressed as percent of labelled Re retained at different time intervals (n=2).*

ascites fluid was collected with a syringe and the peritoneal cavity was washed with PBS, pH 7.4 and the washings were collected. Blood samples were collected by cardiac puncture. Organs were dissected, blotted on filter paper to remove the blood, and placed separately into plastic (K-free) scintillation vials and the radioactivity was counted using a gamma counter. The results were expressed as percent injected dose per gram of each tissue. The cell number in ascites fluid was counted by diluting the sample appropriately. The total ascites fluid was centrifuged and the volume of the supernatant as well as the pellet were noted.

All experimental protocols with animals were pre-approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee using guidelines established by the Canadian Council on Animal Care.

Statistical analysis. The statistical analysis was carried out using the student's *t*-test in Graph Pad InStat 3.05 version software (San Diego, CA). $p<0.05$ was considered significant.

Results

Biodistribution studies were performed in NIH:OVCAR-3 cell xenografted mice after administration of the formulations (^{188}Re, ^{188}Re-B27.1, ^{188}Re-P54) intraperitoneally. The B27.1 (anti CA 125 antibody) and P54 (antibiotin antibody) were photoactivated and labeled with ^{188}Re as described previously. To ensure that the binding of B27.1 antibody to the cancer cells is not non-specific binding, an irrelevant antibody (P54 antibody) that has no specificity to the cancer cells is also used in the present study.

Ultravoilet light was used as a probe in analysis of enzyme function as early as 1970. Sykes *et al*., (31) have photoactivated various monoclonal antibodies and concluded that this technique may be applicable to all antibodies but individual photoactivation process has to be optimized for each antibody. Upon exposure to the UV light, about 80% and 10% of the absorbed dose may be attributed for tyrosine and tryptophan content respectively. The primary mechanism of photoactivation of the antibody is the generation of highly reactive sulphydryl groups. The antibody was tested for immunoreactivity after photoactivation process. The labelling efficiency of both the B-27.1 and P-54 was >90% and enzyme linked immunosorbent assay (ELISA) results (data not shown) confirmed that the immunoreactivity of the antibody was retained after the radiolabeling process. ^{188}Re-labeled antibody was purified from the free ^{188}Re using Sephadex G25 column chromatography as shown in figure 1. These Re-labeled antibodies were used in the biodistribution studies immediately after the purification. The percent of recovery of the ^{188}Re in various organs was calculated at 4 hour time point, which is conventionally assumed to be the maximum time for complete distribution of any carrier system administrated peritoneally.

As shown in figure 2a, accumulation of free ^{188}Re in thyroid and stomach were significantly high comparing with all other organs ($p<0.01$). The percent injected dose per gram of the tissue in thyroid and stomach were 50 and 45% respectively. Except these two organs, recovery of ^{188}Re in other organs was very low with only 5% in solid tumor, and all other organs showed <2% of the injected dose per gram of the tissue.

In the group administered with 188Re-B27.1, about 20% of the injected dose was recovered per gram of the ascites cells (figure 2b). A significantly higher amount of Re was recovered in kidney (8% per gram) followed by ascites fluid and the gastrointestinal tract. Recovery of ^{188}Re in thyroid and stomach was ~2-3% of the injected dose per gram of the tissue. Less than 5% of the injected dose was found in all the other organs. When an irrelevant antibody labeled with ^{188}Re (Re-P54) was administered, only about 6% of the injected dose was accumulated in the ascites cells. The difference in accumulation in gastrointestinal tract (GIT) and ascites cells was not significant. However the highest accumulation, approximately 9% of the injected dose per gram of the tissue, was seen in kidney (figure 2c). The concentration of ^{188}Re in brain was insignificant with both B27.1-Re as well as free rhenium.

Discussion

The present study is a preliminary experiment to confirm the target specificity of the B27.1 antibody to the OVCAR-3 cells *in vivo*. Biodistribution of free rhenium was highest in thyroid and stomach. However none of the other organs

showed any significant accumulation. We could only recover 60-70% of the administered dose on termination of the study. It appears that a large amount of administered dose was excreted, though the exact amount excreted was not accounted.

The extent of ^{188}Re uptake by the ascites tumor cells with B27.1-Re was significantly higher than any other formulation tested in the present study ($p<0.01$). It is evident that the B-27.1 antibody was specifically binding to CA125 antigens present on cancer cell surface. The lower concentrations of ^{188}Re in thyroid and stomach with B27.1-Re as compared to free Re is an indication that ^{188}Re was retained in the peritoneal cavity, precisely in the ascites fluid. However, the recovery of ^{188}Re in kidney was significantly higher with B27.1-Re preparation than the free rhenium preparation. This may be accounted for the slow dissociation of ^{188}Re from the antibody and excretion by kidney following systemic absorption. It may be probable that B27.1-Re may not be stable over the time period used for estimating the biodistribution. Although the biodistribution results with one time point are difficult to interpret, a very high magnitude of B27.1-Re in ascites cells is in agreement with our concept and can be exploited for pretargeting to the ovarian cancer cells.

P-54 antibody labeled with ^{188}Re was used as a negative control in the present study to check if the accumulation of B27.1-Re in ascites cells was not due to non-specific binding to the cancer cells. Interestingly, recovery of ^{188}Re in ascites cells with P54-Re was about 4 fold lower than the B27.1-Re confirming that the rhenium accumulation with B-27.1 antibody was not because of non-specific binding of the antibody to the tumor cells. However, with both the B27.1 and P-54 antibodies, the recovery in thyroid and stomach was lower than the free rhenium preparation. The ^{188}Re levels in kidney with P54-Re was higher than B27-Re. This may be attributed to the stability of Re attached to P54 antibody.

Stability of radiolabeled B27.1 and P54 antibodies was assessed by determining the percent of ^{188}Re retained on a given amount of antibody at different time intervals by size exclusion chromatography. Though Re-labeled antibodies were found stable in PBS pH 7.4 (>90% of the labeled amount retained after 5 hrs), about 20% of labeled ^{188}Re was lost when the radiolabeled antibodies were incubated with cysteine for 5 hrs as shown in figure 3. Currently the preparation of the bispecific antibody by chemical conjugation of P54 antibody and B27.1 antibody is in progress.

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