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A DIFFUSIBLE RESISTANCE FACTOR(S) IN SPONTANEOUS MITOMYCIN RESISTANT MAMMALIAN CANCER CELLS

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ABSTRACT

We developed a Mitomycin C (MMC)-resistant mouse breast cancer cell subline, R-FM3A, from FM3A parent cells (W-FM3A) by continuous exposure to a concentration of 0.01 μg/ml MMC for over two months. R-FM3A cells were resistant to MMC and decreased in response to the combined effects of ionizing irradiation and MMC. The resistance to MMC and ionizing irradiation was mediated by a cell-free medium, in which R-FM3A cells were cultured for 12 hours without MMC (Re-medium). The cell cycles measured by means of a flow cytometer were not influenced by the Re-medium when W-FM3A cells were treated by MMC. But the cell cycles measured by means of a flow cytometer were influenced by the Re-medium when W-FM3A cells were irradiated at a dose of 4 Gy.

Key Words: Mitomycin C, Diffusible resistance factor, FM3A, Flow cytometer

INTRODUCTION

A significant problem in clinical treatment that uses anti-cancer chemotherapy is resistance of cancer cells to the drugs. Cancer cells sometimes develop resistance to other anticancer drugs or ionizing radiation.[1-4] This resistance, cross-resistance to drugs, and radioresistance are suspected as factors which prevent the control of malignant tumors. To overcome such resistance, malignant tumors are treated using multi-modal therapy, and new anti-cancer drugs are developed. There have been previous reports concerning the mechanism of resistance against anti-cancer drugs and radiation using human malignant tumors or spontaneously resistant cell lines.[3-6] The sensitivity to Mitomycin C (MMC) has influenced treatment of NaBu,[2] Glutathione / Glutathione transferase level,[7] and intracellular quinone oxidoreductase activity.[8] There have been two reports about the diffusible resistance and cross-resistance factors (DRF) of melanoma subclones.[9,10] We developed a MMC-resistant mouse breast adenocarcinoma cell subline, R-FM3A, from FM3A parent cells by exposuring them to a concentration of 0.01 μg/ml MMC for two months. This paper has three goals. The first is to evaluate the existence of DRF in an adenocarcinoma cell line. The second is to evaluate the influence of DRF on the combined effect of ionizing irradiation and MMC. The third is to evaluate the influence of DRF on the cell cycle after MMC treatment and ionizing irradiation. There have been no previously published reports examining the influence of DRF on the cell cycle.

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MATERIALS AND METHODS

The FM3A cell line (W-FM3A), originally established from a spontaneous C3H mouse breast cancer and maintained continuously as a suspension culture, was cultured at 37 ± 0.5°C in Eagle's MEM supplemented 10% calf serum, 0.3 mg/L of Glutamine and 0.12% $NaHCO_3$ as a buffer (the Medium). Under these conditions, the population doubling time was 12 ± 0.5 hours. The spontaneous MMC resistant FM3A cell line (R-FM3A) isolated in our department was used. The R-FM3A cells grew from the W-FM3A cells. W-FM3A cells were cultured in the Medium containing 0.01 μg/ml MMC at 37 ± 0.5°C. The Medium was changed every 48 hours. After two months exposure to MMC, W-FM3A cells showed resistance to MMC. The R-FM3A cells were isolated from the colonies by five serial extractions. The R-FM3A cells showed no change in resistance to MMC after being cultured in the Medium without MMC during the period of examination. In this paper, used R-FM3A cells were maintained in the Medium with 0.01 μg/ml of MMC. Immediately before the experiments, W-FM3A and R-FM3A cells were rinsed twice by the freshly prepared Medium. The Re-medium was obtained as follows. R-FM3A cells were cultured in the Medium without MMC for 12 hours. The R-FM3A cells were removed by centrifugation at 1500 rpm for six minutes twice. One volume of this cell free medium was mixed with nine volumes of freshly prepared Medium. The resultant medium refers to the Re-medium (Fig. 1). The W-Re-FM3A cells consisted of rinsed W-FM3A cells suspended in Re-medium. These three cell lines (W-FM3A, R-FM3A, and W-Re-FM3A) were cultured in suspension. Cell survival was equated with colony forming ability.[11,12] In brief, the cells were placed into 0.4% agar with MEM containing 20% fetal bovine serum (GIBCO) and incubated at 37 ± 0.5°C for ten days. The colonies containing more than 50 cells were counted.

Experiment 1. Cell Growth Rate

W-FM3A, R-FM3A, and W-Re-FM3A cells were suspended in the Medium. The number of cells was 10^4/ml initially. The W-FM3A and W-Re-FM3A cells were cultured without MMC. The R-FM3A cells were cultured in the Medium containing 0.01 μg/ml MMC. The number of cells was measured every six hours using a Haematocytometer (Thoma, NITIRIN, Japan).

Experiment 2. Cell Survival Rate after exposure to MMC for six or 24 hours

MMC was dissolved in the Medium. The concentrations of MMC added to the Medium in these experiments were 0.01, 0.02, 0.05, 0.1, 0.2, and 1.0 μg/ml. The Medium in the test tube was replaced with the Medium containing MMC (Fig. 1). The cells were incubated for six or 24 hours. After incubation, the medium was removed and the cells were rinsed in fresh Medium. Cell survival was equated with colony forming ability,[11,12] and every examination was repeated three times. Each set of data was compared with W-FM3A using Stutent's t-test. When the P value was less than 0.05, we concluded that there was a significant difference.

Experiment 3. The combined effect of MMC and radiation on cell survival

Irradiation was performed using a 4MV linear accelerator (MEVATRON, MLC-10) at a dose rate of 0.3 Gy/min. Irradiation doses were 1, 2, 3, 4, and 6 Gy. The three cell lines were suspended in a test tube immediately before irradiation in the Medium containing 0.01 μg/ml MMC (Fig. 1). After irradiation, the medium was removed and the cells were rinsed in fresh Medium. Cell survival was equated with colony forming ability.[11,12] Every examination was repeated three times and each set of data was compared with the cell survival without MMC (Control) using Stutent's t-test. When the P value was less than 0.05, we concluded that there was a significant difference.

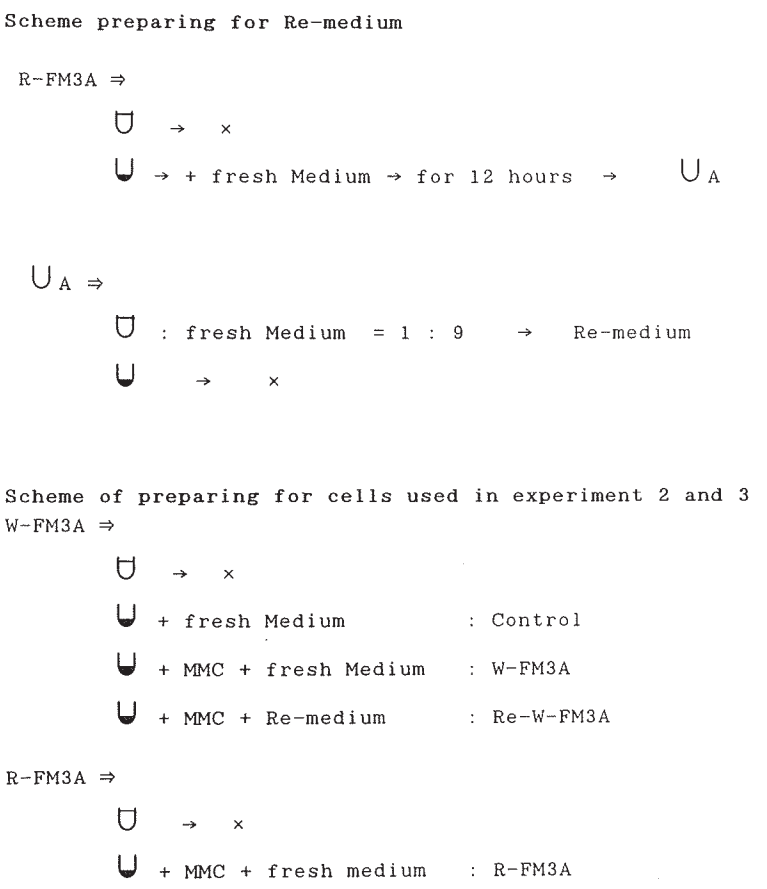

Figure 1. Scheme preparing for Re-medium and for used cells in experiments 2 and 3.
∪ : cell free medium, ⊍ : cells, × : pour out
$\cup_A$: Cultured R-FM3A cells in MMC free Medium for 12 hours
⇒ : cells were removed by centrifugation at twice 1500 rpm for six minutes.

Experiment 4. Influence of MMC treatment and ionizing irradiation on the cell cycle of the three cell lines

The three cell lines were suspended in a test tube in the freshly prepared Medium containing 0.01 μg/ml MMC. After treatment with MMC, the cells were cultured at 37±0.5°C for 24 hours. The amount of intracellular DNA was measured using a flow cytometer (JNM-GSX-270, Electronics Co. Ltd., Japan). Cells were fixed by 70% ethanol, treated with 1 mg/ml RNase, stained with 50 μg/ml propidium iodide (Signa Chemicals, St. Louis, MO, USA), then counted using a flow cytometer. The data was analyzed by the Multicycle Software cell cycle analysis program.[13)]

The three cell lines were suspended in a test tube immediately before irradiation in the Medium without MMC. Irradiation was performed using a 4MV linear accelerator at a dose rate of 0.3 Gy/min. The irradiation dose was 4 Gy. After irradiation, the cells were cultured at 37±0.5°C for 24 hours. The amount of intracellular DNA was measured using a flow cytometer.

RESULTS

Experiment 1. Cell Growth Rate

PDT (population doubling time) was 12 ± 0.5 hours for the three cell lines.

Experiment 2. Cell Survival Rate after exposure to MMC for six or 24 hours

The R-FM3A cells showed significantly higher survival rates than the W-FM3A cells following both six and 24 hours of treatment with MMC. The survival rate of the W-Re-FM3A cells was intermediate between that of the R-FM3A and the W-FM3A cells (Fig. 2).

Experiment 3. The combined effect of MMC and radiation on cell survival

The survival curve of the W-FM3A cells exposed to irradiation without MMC in the Medium was the same as that of the R-FM3A cells with 0.01 μg/ml of MMC in the Medium. The survival of the W-FM3A cells after irradiation with 0.01 μg/ml of MMC in the Medium was significantly decreased compared to the Control at the doses of 3, 4, and 6 Gy. The survival of the W-Re-FM3A cells after irradiation with 0.01 μg/ml of MMC in the Medium was significantly increased compared to the Control at the dose of 3, and 4 Gy (Fig. 3).

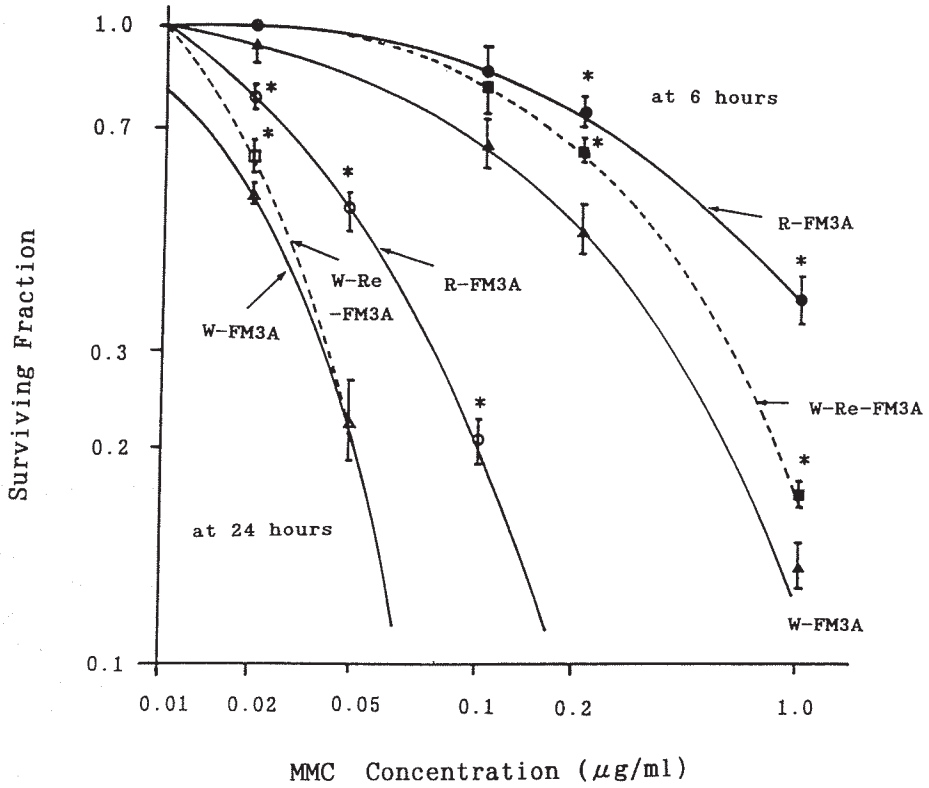

Figure 2. Survival curve after exposure to MMC. Open symbols represent a treatment time of 24 hours. Closed symbols show a treatment time of 6 hours. There were three replications. Bars show the mean ± SD. *: Significant difference between groups, $p < 0.05$.

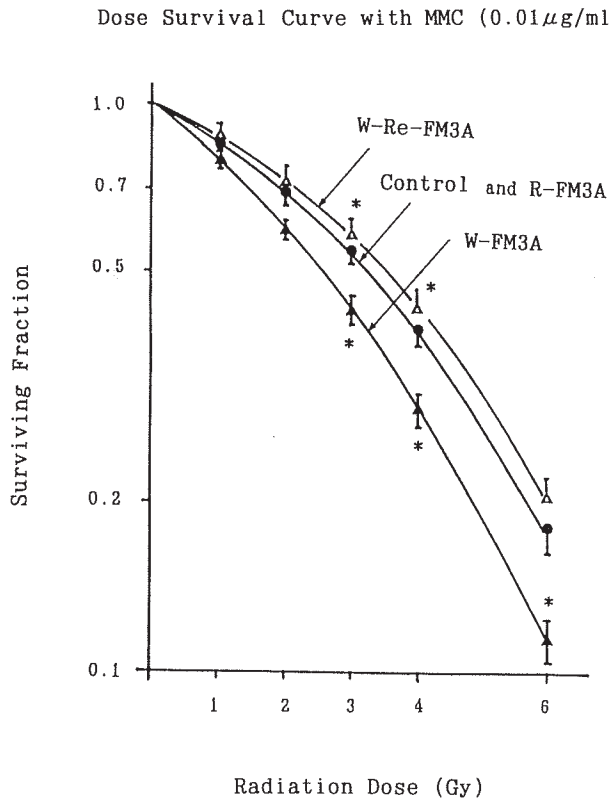

Figure 3. Survival curve after combined exposure to MMC and ionizing irradiation. There were three replications. Bars show the mean ± SD. ✻: Significant difference between groups, $p < 0.05$.

Experiment 4. Influence of MMC treatment and ionizing irradiation on the cell cycle of the three cell lines

There was no difference among the histograms of the three cell lines treated with 0.01 μg/ml of MMC for 24 hours. The influence of Re-medium on the cell cycle was not found (Fig. 4). The G1 phase of the W-FM3A cells decreased after irradiation. Statistical analysis was not performed because of little data. There were no differences in the histograms between the R-FM3A cells and the W-Re-FM3A cells (Fig. 5). The Re-medium had a influence on the cell cycle of the irradiated W-FM3A cells.

DISCUSSION

Some cultured cell lines manifested transient resistance to MMC, and cross resistance to chemicals or radiation after continuous exposure to low concentrations of MMC.[5,6] We developed a MMC resistant FM3A cell line (R-FM3A) by exposure to 0.01 μg/ml MMC for two months; 20% of the parent FM3A cells died by exposure to 0.01 μg/ml of MMC over 24 hours. The R-FM3A cells survival was not decreased by the same treatment. The FM3A cells were known for their ability to spontaneously change their sensitivity to chemicals and

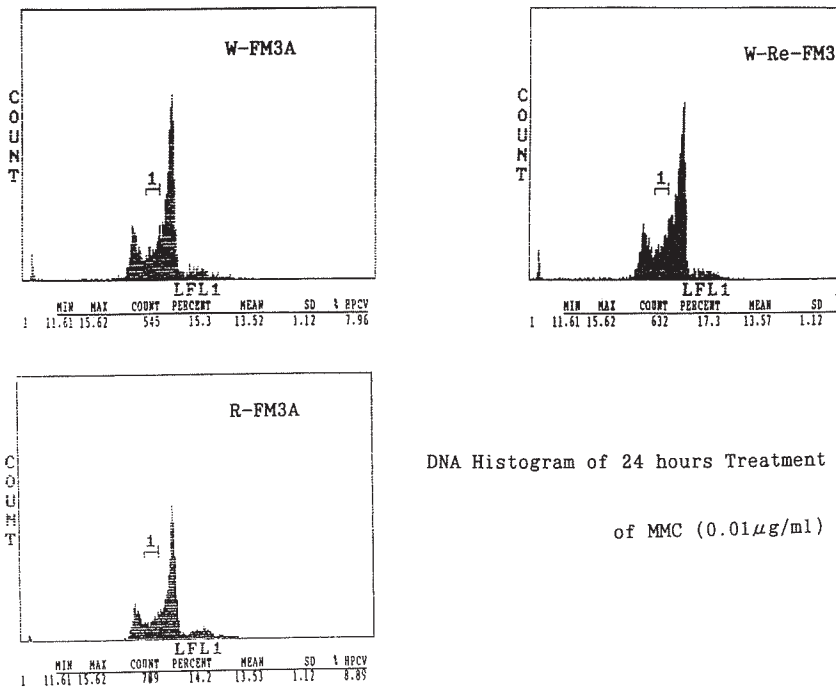

Figure 4. DNA histograms of three cell lines treated with 0.01 μg/ml of MMC for 24 hours.

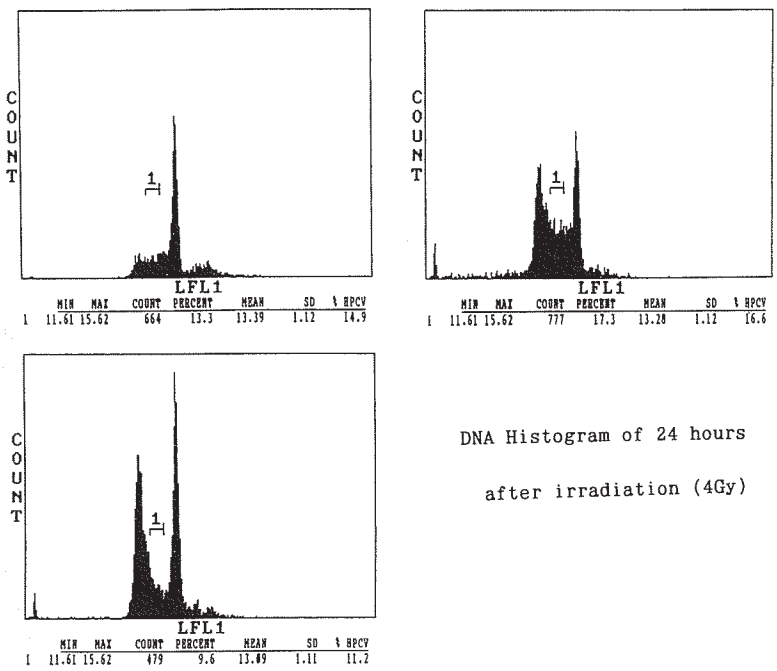

Figure 5. DNA histograms of three cell lines after 24 hours of 4 Gy irradiation.

radiation.[3,4] MMC toxified cultured mammalian cells by influencing the intracellular DNA,[14] and activated spontaneous MMC resistance.[2] The resistance to MMC of the R-FM3A cells showed no change during the experimental time. The resistance of R-FM3A was stable after being cultured in a MMC-free Medium for six months. The MMC resistance over the six months in the MMC free Medium shows that the resistance is not from an intracellular pH change or from a change in the membrane permeability. In this paper, used R-FM3A cells were maintained in the Medium with 0.01 μg/ml of MMC. The cell cycle and PDT of R-FM3A were not different from the parent FM3A. The mechanism of MMC resistance was suspected to be separate from DNA synthesis.

The cell free medium, in which R-FM3A cells had been cultured for 12 hours, made parent FM3A cells resistant to MMC (Re-medium). The transference of DRF through the medium was suspected to be the reason for the resistance of FM3A cells in the Re-medium. But MMC resistant R-FM3A cells in a freshly prepared medium showed more resistance to MMC. We suspected that the active intracellular enzymes or ectoenzymes from MMC resistant mutant cells was the DRF.

There have been two papers about DRF from MMC resistant subclone of melanoma cells.[9,10] In these two papers, the presence of a diffusible substance was not demonstrated. In this paper, the resistant cell line was a mutant from parent FM3A cells. This report is the first to show that a newly isolated mutant cell line has DRF.

In a previous report,[15] we showed that a Re-medium did not have an influence on the cell cycle of W-FM3A cells. In this paper, we reported that DRF did not have an influence on the cell cycle after treatment by 0.01 μg/ml of MMC for 24 hours, and that DRF had an influence on the cell cycle after ionizing irradiation. There may be different mechanisms associated with resistance to MMC and cross-resistance to ionizing irradiation. Resistance to MMC was not caused by cell cycle changes, and the survival rate of the W-Re-FM3A cells was between that of the R-FM3A and the W-FM3A cells after treatment by MMC. We speculated resistance to MMC was primarily dependent on the amount of DRF. Resistance to ionizing irradiation was more complex. The survival of the W-Re-FM3A cells after irradiation with 0.01 μg/ml of MMC in the Medium was significantly increased compared to R-FM3A cells at the doses of 3 and 4 Gy. DRF in the Re-medium made W-FM3A cells more resistant to ionizing irradiation than R-FM3A cells. MMC treatment before ionizing irradiation made cultured cells resistant to ionizing irradiation.[16] It was not clear whether DRF was extracted from W-FM3A cells or not.

The combined effect of MMC and ionizing irradiation, especially with respect to time, was reported in 1965.[16] The combined effect, when MMC treatment was simultaneous or before ionizing irradiation, was greater than that when radiation was followed by MMC treatment. In our previous report,[15] the combined effect with 0.01 μg/ml of MMC and ionizing irradiation was greater than that with 0.1 or 1.0 μg/ml of MMC when the two treatments were simultaneous. In this paper, the main purpose was to evaluate the effect of Re-medium on W-FM3A cells. Simultaneous combinations of 0.01 μg/ml of MMC and ionizing irradiation were measured. W-FM3A cells showed a synergistic effect when simultaneously treated with MMC and ionizing irradiation. These results were the same as those in previous reports.[15,16] R-FM3A cells showed the same survival rate after simultaneous treatment of MMC and ionizing irradiation as the survival rate of W-FM3A cells after ionizing irradiation without MMC (Control). R-FM3A cells showed a decreased synergistic effect following simultaneous treatment with MMC and ionizing irradiation. The survival of W-FM3A cells in Re-medium (W-Re-FM3A) after simultaneous treatment of MMC and ionizing irradiation was higher than in the Control. W-Re-FM3A cells showed more resistance to ionizing irradiation than R-FM3A cells. The reason why W-Re-FM3A cells showed more resistance to ionizing irradiation than R-FM3A cells was not clear.

We speculate that R-FM3A cells have the same radio-sensitivly as W-FM3A cells under 0.01 μg/ml of MMC, and that the Re-medium has cross-resistance to ionizing irradiation.

Clinical neoplasms are not uniform hereditary genes like cultured cell lines.[17] Clinical neoplasms are maintained in various culture conditions, such as variable pH and oxygen pressure. Their sensitivity to chemicals or radiation is variable, therefore their potential to become spontaneously resistant is also variable. Resistance was induced as a direct response of the tumor cells to the antineoplastic drug. Mutation that leads to drug resistance often occurs. The most explored drug effects involve the expression of the multi-drug resistance gene, an outward transport system, and confluence dependent resistance, which blocks the passive transmembrane drug transport. Once tumor cells become resistant, the resistance factor influences the existence of DRF, which may be a significant factor that causes the failure of treatments for malignant diseases. Chemoresistance and cross resistance to ionizing irradiation after a series of chemotherapy treatment is a factor that inhibits the control of malignant neoplasms. From our results, the entire tumor may be resistance to MMC and ionizing irradiation under the influence of DRF from resistant cells when a small part of the tumor gains resistance to MMC. Insufficient chemotherapy makes it difficult to control malignant neoplasms. We suspected that DRF was one of the reasons for failure in previous clinical trials with MMC.[18,19] Further experimentation is needed to detect the chemicals that cause DRF.

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