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Augmentation effects of lymphocyte activation by antigen-presenting macrophages on FDG uptake

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Objective: Research on FDG-uptake by blood cells has revealed that FDG is incorporated by macrophages and granulocytes, as well as activated lymphocytes. These characteristics of FDG suggest the possibility of visualizing the distribution of immunocytes in target organs. The aim of this study was to investigate if mouse spleen-derived lymphocytes, activated by macrophages presenting sheep red blood cell (sRBC) antigens, could be traced by FDG. ***Methods:*** One percent of a sRBC suspension was injected into the peritoneal cavity of mice thereby creating immunity to the sRBC antigen. The splenocytes, consisting mostly of lymphocytes, were isolated, and serum containing the anti-sRBC antibody was mixed with sRBC to prepare sRBC-antibody complexes (sRBC-AbCs). Then five percent of a thioglycolate medium was injected into the peritoneal cavity of the same mice, and macrophages of ascitic cell origin were obtained. These macrophages were added to the sRBC-AbCs to induce sRBC antigen presenting macrophages. These were incubated with splenocytes obtained from sRBC immunized mouse (sRBC immunized splenocytes) or non-immunized splenocytes to induce a T cell immune response. [^{3}H]deoxyglucose ([^{3}H]DG) and FDG were incorporated in splenocytes, and the quantity of their uptake was measured. ***Results:*** [^{3}H]DG uptake by sRBC-immunized splenocytes was about eleven times as high as that of non-immunized splenocytes. In contrast, [^{3}H]DG uptake by sRBC-immunized splenocytes, co-cultured with macrophages phagocytizing sRBC-AbCs, was about 40 times higher compared with non-immunized splenocytes. Splenocytes in non-immunized mice picked up very little [^{3}H]DG, despite co-culture with macrophages phagocytizing sRBC-AbCs. Similar tendencies were observed with FDG. ***Conclusions:*** These results suggest that the SUV calculated in PET reflects not only the number of lymphocytes, but also the activation state of the lymphocytes themselves. In addition, the biodistribution of antigen specific lymphocytes, that have been taken up FDG *in vitro* and returned to the body, can be observed through PET.

Key words: lymphocyte, macrophage, FDG-uptake, sRBC immunized splenocyte

INTRODUCTION

RECENTLY, positron emission tomography (PET) has been developed as a novel nuclear medicine diagnostic procedure, and clinically applied to tumor diagnosis.[1] ^{18}F-2-deoxy-2-fluoro-D-glucose (FDG) is a radioactive drug that is widely used for this purpose. Since glucose metabolism is increased in tumor cells, the glucose analog FDG is specifically taken up by them.[2] FDG PET is an imaging technique for visualizing increased glucose metabolism in such tumor cells, and has a higher sensitivity and a higher spatial resolution than conventional single photon emission tomography (SPECT).[3,4] It has been reported that FDG PET is useful because of its higher diagnostic accuracy for post-therapeutic residual or recurrent lung, colorectal, and head and neck tumor than

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that of CT and MRI.[5–7] However, many cases have been reported in which FDG is taken up not only by malignant tumors but also by benign tumors and inflammatory lesions,[8,9] reducing the specificity of FDG PET. It is very likely that in inflammatory lesions, FDG is taken up by immunocompetent cells, particularly NK cells, macrophages, and T cells. In fact, Rau et al.[10] made a mouse model of acute lymphadenitis by inoculating concanavalin A and a metastatic lymph node from a mouse inoculated with T cell lymphoma cells, examined the FDG uptake by inflammatory or metastatic lymph nodes, and showed that FDG was taken up not only by lymphoma cells but also by macrophages and granulocytes. On the other hand, if these characteristics of FDG could be utilized for investigating the localization and kinetics of immunocompetent cells circulating through the tissues, it would provide much information for the elucidation of the pathogenesis of various diseases. Bushart et al.[11] have shown that when the lymphocyte cell line IM9 enters the cell division phase, ^{3}H-deoxyglucose uptake increases, permitting FDG incorporation in these cells. However, these results do not indicate that cells activated physiologically by specific antigens can taken up FDG. Macrophages phagocytize viruses, fragments of cells damaged by viral infection, antigens shed and secreted by tumor cells, and fragments of tumor cells injured by NK cells, and present antigens to T cells. This antigen presentation is the major function of macrophages and essential for T cell clone formation. However, the details of FDG uptake by these macrophage–activated T cells remain obscure. This study aims to investigate the possibility of FDG incorporation in mouse spleen-derived lymphocytes that have been activated physiologically by macrophages presenting sheep red blood cell (sRBC) antigens.

MATERIALS AND METHODS

1. FDG uptake by human peripheral blood mononuclear cells in vitro

Two m*l* blood of a 25-year-old male healthy volunteer was drawn into a heparin-coated syringe to analyze the uptake of FDG by lymphocytes. The blood was diluted with an equal volume of phosphate-buffered saline (PBS), overlaid with 5 m*l* of sodium metrizoate-Ficoll for human lymphocytes, and centrifuged at 1,200 rpm for 30 minutes. The fraction consisting of the peripheral blood mononuclear cells (PBMCs) was washed with PBS three times, and maintained in static culture in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA) at 37°C in an atmosphere of 5% CO_2. Four hours later, the plastic-adherent cells were removed, and PBMCs (5×10^5 cells/m*l*) consisting mostly of lymphocytes were isolated. To induce lymphocyte division, a polyclonal mitogen, phytohemagglutinin (PHA) (Sigma-Aldrich Co., St. Louis, USA), was added to the PBMCs, which were incubated for 2 days. After FDG (740 kBq/m*l*) was administered, the PBMCs were incubated for 60 minutes, washed three times with PBS containing 0.1% glucose, and measured for FDG uptake in a gamma counter (ARC-2000, Aloka, Tokyo).

2. Preparation of immunized mice and antibody to sRBC

Three weekly intraperitoneal injections of 0.2 m*l* of a 1% sRBC (Toyo Bio, Tokyo) suspension were given to mice (C57BL/6J) to make them immune to sRBC antigen. One week later, the spleen was removed, and blood was collected. The spleen was minced in cold PBS, and filtered through a stainless steel mesh to prepare a suspension of isolated splenocytes. The splenocytes were washed with PBS three times, and maintained in static culture in RPMI 1640 medium supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. Four hours later, the plastic-adherent cells were removed, and splenocytes consisting mostly of lymphocytes were isolated. Splenocytes obtained as described above from mice not immunized with sRBC served as controls. The quality of the separated lymphocytes was good with >90% yield confirmed by flow cytometry. On the other hand, blood was centrifuged at 3,000 rpm for 15 minutes, and the serum containing anti-sRBC antibody was isolated, and inactivated by heating at 56°C for 30 minutes.

3. Preparation of mouse macrophages

Mice were intraperitoneally injected with 0.5 m*l* of 5% thioglycolate medium (Nissui Pharmaceutical Co., Tokyo), and kept for 3 days. After the mice were sacrificed, 7 m*l* of cold PBS was injected into the peritoneal cavity, and the ascitic fluid was collected. The ascitic cells were washed with PBS twice, suspended in RPMI 1640 supplemented with 10% FBS, and maintained in static culture for 2 hours at 37°C in an atmosphere of 5% CO_2. The nonadherent cells were removed, and macrophages were isolated.

4. Induction of sRBC antigen-presenting macrophages and their interaction with splenocytes

Induction of sRBC antigen-presenting macrophages and activation of antigen specific lymphocytes were prepared according to the method described by Kruisbeek[12] and Harding.[13] The lowest dilution of the above (2) obtained anti-sRBC antibody-containing serum that did not agglutinate sRBCs was mixed with an equal volume of 1% suspension of sRBCs, and the mixture was shaken at 37°C for 60 minutes, followed by washing with PBS three times to prepare sRBC-antibody complexes (sRBC-AbCs). These sRBC-AbCs were mixed with the macrophages obtained above at a mixing cell ratio of 10 : 1 (3), and the mixture was incubated for 20 hours at 37°C in an atmosphere of 5% CO_2 to induce sRBC antigen-presenting macrophages, which were inactivated by 4 MV X-irradiation (Clinac-2100C, Varian Medical Systems, CA, USA)

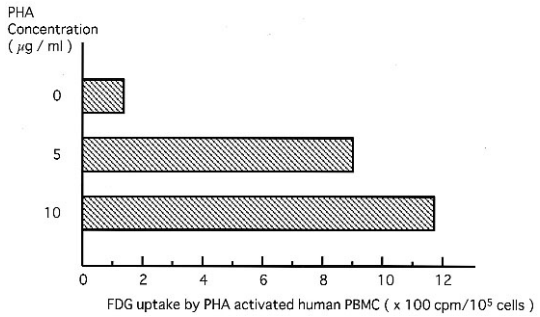

Fig. 1 Phytohemagglutinin (PHA), which induced division of T cell, was added to the PBMCs. After FDG was administered, the PBMCs were incubated for 60 minutes and measured for FDG uptake. Each bar indicates the mean radionuclide uptake of triplicate samples.

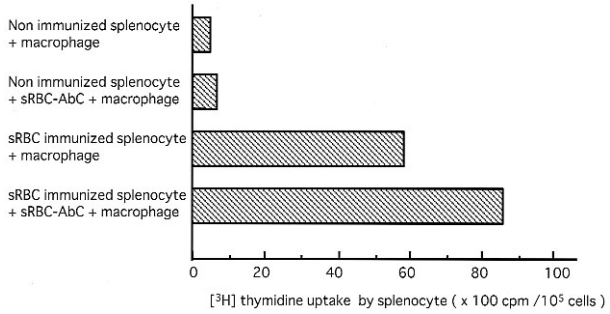

Fig. 2 [^{3}H]thymidine uptake by splenocytes obtained from non-immunized and immunized mice with sRBC. Each bar indicates the mean radionuclide uptake of triplicate samples. sRBC: sheep red blood cell, sRBC-AbC: sRBC-antibody complex

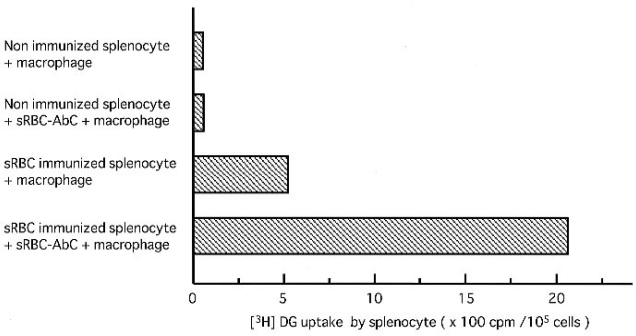

Fig. 3 [^{3}H]deoxyglucose ([^{3}H]DG) uptake by splenocytes obtained from non-immunized and immunized mice with sRBC. Each bar indicates the mean radionuclide uptake of triplicate samples.

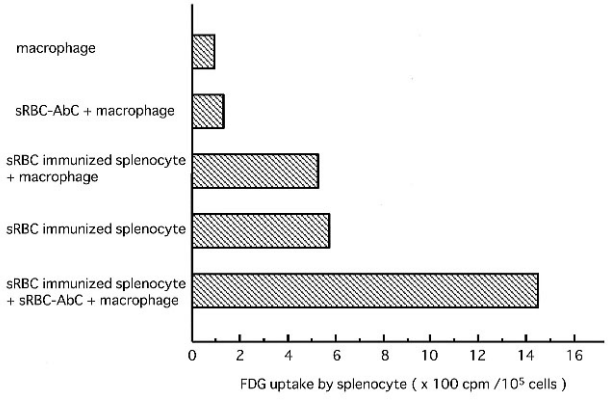

Fig. 4 FDG uptake by mouse macrophages and splenocytes obtained from immunized mice with sRBC. Each bar indicates the mean radionuclide uptake of triplicate samples.

with a dose of 20 Gy. These sRBC antigen-presenting macrophages (1×10^4 cells/m*l*) were incubated for 2 days with splenocytes obtained from sRBC-immunized mouse (sRBC-immunized splenocytes) or non-immunized splenocytes (5×10^5 cells/m*l*) obtained above (2) to induce the immune response of the T cells in the splenocytes. Subsequently, [^{3}H]thymidine (3.7 kBq/m*l*), [^{3}H]deoxyglucose ([^{3}H]DG) (3.7 kBq/m*l*) (Amersham Pharmacia Biotech, Tokyo), or FDG (740 kBq/m*l*) was administered for 16, 1, and 1 hour, respectively. After the termination of incubation, the cells were washed three times with PBS supplemented with 0.1% glucose, and the amount of radionuclide uptake was measured in a liquid scintillation counter (LSC-1000, Aloka, Tokyo) or gamma counter. Radionuclide uptake was measured in triplicate sets of cells, and the mean radionuclide uptake was evaluated.

RESULTS

Cell division-inducing PHA was administered to activate lymphocytes *in vitro* (Fig. 1). PHA (5–10 μg/m*l*) was administered to PBMCs consisting mostly of lymphocytes and, after incubation for 48 hours, FDG was allowed to act on the cells for 60 minutes. As a result, FDG uptake increased, depending on the concentration of PHA: by about 8 times (11.8×10^2 cpm) at 10 μg/m*l* of PHA compared with a control (1.4×10^2 cpm) receiving no PHA. There was no appreciable difference in cell number between control and PHA-stimulated PBMCs during the first 48 hours.

Next, we tried to activate splenocytes with macrophages that were ready to present antigen. As a sign of splenocyte proliferation, [^{3}H]thymidine uptake was measured (Fig. 2). To observe glucose metabolism, [^{3}H]DG uptake was also measured (Fig. 3). sRBC-immunized mouse splenocytes as antigen-specific cells were incubated with mouse macrophages that had been allowed to phagocytize sRBC-AbCs and had become ready to present antigen. As controls, the following co-cultures were performed: [1] co-culture of non-immunized splenocytes with macrophages phagocytizing no sRBC-AbCs, [2] co-culture of non-immunized splenocytes with macrophages phagocytizing sRBC-AbCs, and [3] co-culture of sRBS-

immunized splenocytes with macrophages phagocytizing no sRBC-AbCs. The same cell number of splenocytes was used for each group. The non-immunized splenocytes used as controls [1] and [2] incorporated very little [^{3}H]thymidine, 5.0×10^2 cpm and 6.8×10^2 cpm, respectively. In contrast, sRBC-immunized splenocytes used as control [3] incorporated about 11 times as much [^{3}H]thymidine (58.6×10^2 cpm) as the splenocytes used as control [1], indicating that sRBC immunization alone activates splenocytes. [^{3}H]thymidine uptake by sRBC-immunized splenocytes activated by sRBC antigen-presenting macrophages was found to be about 13 times as high (86.0×10^2 cpm) as that by splenocytes used as control [2], indicating that sRBC-immunized splenocytes become more activated by sRBC antigen-presenting macrophages. The number of splenocytes did not increase by adding macrophages phagocytizing no sRBC-AbCs or sRBC antigen-presenting macrophages during the first 48 hours. On the other hand, [^{3}H]DG uptake showed a similar tendency to that of [^{3}H]thymidine uptake. [^{3}H]DG uptake by splenocytes in controls [1] and [2] was together very low at 0.5×10^2 cpm. In contrast, [^{3}H]DG uptake by sRBC-immunized splenocytes in control [3] was about 11 times (5.3×10^2 cpm) as high as that by splenocytes in control, [1] and [^{3}H]DG uptake by sRBC-immunized splenocytes co-cultured with macrophages phagocytizing sRBC-AbCs was about 40 times higher (20.7×10^2 cpm) compared with non-immunized splenocytes in control, [2] and about four times as high as that with macrophages phagocytizing no sRBC-AbCs in control [3].

Next, as described above, the FDG uptake by sRBC-immunized splenocytes co-cultured with sRBC antigen-presenting macrophages was measured (Fig. 4). The following controls were used: [4] macrophages phagocytizing no sRBC-AbC, [5] macrophages phagocytizing sRBC-AbCs, [6] co-culture of sRBC-immunized splenocytes with macrophages phagocytizing no sRBC-AbCs, and [7] sRBC-immunized splenocytes alone. We found that little or no FDG was taken up by macrophages in controls [4] and [5] as evidenced by radioactivities of 1.1×10^2 cpm and 1.3×10^2 cpm, respectively. In contrast, the FDG uptake in controls [6] and [7] was 5.3×10^2 cpm and 5.7×10^2 cpm, respectively, indicating that FDG is easily taken up by sRBC-immunized splenocytes. Moreover, co-culture of these immunized splenocytes with sRBC antigen-presenting macrophages increased FDG uptake by about three times (14.5×10^2 cpm) that in control [6].

DISCUSSION

FDG may be taken up not only by brain cells but also by activated immunocompetent cells. Gallagher et al.[14] observed FDG uptake by blood cells, and confirmed that FDG was taken up by granulocytes, non-activated lymphocytes, monocytes, platelets, and erythrocytes. However, in their study, they did not observe FDG uptake by antigen-specifically activated lymphocytes. In this study, we focused our attention on cellular immunity, which plays an important role in infection and malignancy, and conducted a fundamental study on FDG uptake by antigen-specific lymphocytes, particularly T cells.

Since the majority of PBMCs in the body are in the resting phase, it was anticipated that the uptake rate of FDG would be low. Therefore, we tried to activate PBMCs *in vitro* and incorporate FDG into them. Since PBMCs consist chiefly of monocytes and lymphocytes, we isolated PBMCs consisting of lymphocytes by exploiting the plastic adherence of monocytes. Lymphocytes begin to proliferate on activation by specific antigen or polyclonal mitogens. The PHA used in this study is a kind of polyclonal mitogen, activating T cells (among lymphocytes), which play a central role in cellular immunity. PHA induces the same mechanism of T cell proliferative response as antigen, and allows cells to progress rapidly from the G0 through G1 to S phase.[15] In the current study, the activation of PBMCs with PHA increased FDG uptake by about 8 times. This presumably resulted from T cells entering the cell division cycle and taking up glucose as a source of energy. Therefore, we observed FDG uptake by splenocytes, which are activated via the macrophage, a kind of antigen-presenting cell. We used sRBCs as a specific antigen, and allowed them to react with a previously prepared anti-sRBC antibody to opsonize them. This resulted in the coating of sRBCs with antibody, ultimately facilitated their phagocytosis by macrophages, and encouraged the expectation that the antigen would be presented on the membrane of macrophages. sRBC-immunized lymphocytes bind to these antigen-presenting macrophages, and become activated. To confirm the progress of lymphocytes into the cell division phase, [^{3}H]thymidine taken up in the S phase was used. When sRBC-immunized lymphocytes isolated from splenocytes were allowed to interact with sRBC antigen-presenting macrophages, [^{3}H]thymidine uptake was augmented, which showed the entry of the lymphocytes into the DNA synthesis phase. The possibility that [^{3}H]thymidine uptake was augmented because the number of cells may have doubled during the progress from the M to G1 phase was excluded by the prior confirmation that little or no change occurred in the number of cells within 78 hours of culture. We found that [^{3}H]DG was taken up by mouse splenocytes after immunization of mice by intraperitoneal injection of sRBCs. Furthermore, the uptake of [^{3}H]DG by these splenocytes activated via these antigen-presenting macrophages increased by about four times compared with that via control macrophages. Similar results were observed with FDG.

The splenocytes used in our study were composed of B cells involved in humoral immunity and T cells involved in cellular immunity. Therefore, [^{3}H]DG and FDG may have been taken up not only by activated T cells but also

by activated B cells. However, FDG uptake by immunized splenocytes (Fig. 4, control 3) was only about one-third of that by splenocytes activated by sRBC antigen-presenting macrophages at least. Therefore, presumably the remaining two-thirds of the FDG uptake was by T cells activated further by macrophages.

Kubota et al.[16] reported that FDG accumulated not only in tumor cells but also in young granulation tissue and macrophages, with non-neoplastic cellular elements accounting for approximately 24% of the FDG uptake by cancer tissue, and pointed out that, in reading PET images, FDG uptake by these elements was not negligible. On the other hand, lymphocytes infiltrating inflamed tissues and benign tumors consist chiefly of B cells, whereas T cells far more predominantly infiltrate cancer tissues such as breast cancer,[17] stomach cancer,[18] and oral cancer.[19] This suggests that in these tissues, T cells are activated by secreted antigens from antigen-presenting macrophages and tumor cells. It has been reported that in FDG PET of the head and neck, FDG accumulates in the palatine tonsil, a lymphoid tissue,[20] presumably reflecting uptake by lymphocytes. In general, the amount of FDG uptake that is visualized by PET is evaluated in terms of the standardized uptake value (SUV).[21] In the current study, physiological activation of lymphocytes via antigen-presenting macrophages increased [^{3}H]DG uptake by approximately 40 times, suggesting that the SUV calculated from PET closely reflects not only the number of tumor cells and lymphocytes but also the activation state of lymphocytes themselves. The results obtained in this study indicate that the distribution of antigen-specific lymphocytes that have been taken up FDG *in vitro* and returned to the body can be observed by PET. Although it may be difficult to detect their distribution with the currently available PET devices, it is hoped that a scanner with high sensitivity and high resolution will be developed in the future.

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