Studies of Allogeneic Bone Marrow and Spleen Cell Transplantation in a Murine Model Using Ultraviolet-B Light

By D.H. Pamphilon, A.A. Alnaqdy, V. Godwin, A.W. Preece, and T.B. Wallington

Ultraviolet irradiation inhibits alloreactive and mitogen-induced responses and might reduce both graft-versus-host and host-versus-graft reactions after bone marrow transplantation (BMT). We have studied proliferative responses to mitogens and reactivity in mixed lymphocyte culture after irradiation with ultraviolet (UV)-B light using splenocytes from Balb/c (H-2d) and CBA (H-2k) mice. Response to mitogens and in MLC was strongly inhibited by 20 J/m² and abolished at 50 J/m². Clonogenic cell recovery (CFU-GM; CFU-S) after UV-B irradiation was also reduced. When bone marrow and spleen cells were transplanted from parent (Balb/c) animals into F1 hybrid (Balb/c x CBA) recipients, all animals died with features indicative of graft-versus-host disease (GVHD) in 34 days. If the grafts were first irradiated with 100 J/m² of UV-B at a mean wavelength of 310 nm, then 76% survived to day 80 when they were killed and shown to have normal marrow cellularity. The remainder died in marrow aplasia or of GVHD. H-2 typing in a group of surviving recipients showed either donor hematopoiesis only (8 of 15), mixed allogeneic chimerism (5 of 15), or recipient type hematopoiesis (2 of 15). Higher doses (200 to 300 J/m²) were detrimental to survival with 88% of recipients dying in marrow aplasia. Syngeneic BMT in Balb/c mice showed slower hematopoietic reconstitution when the grafts were first irradiated with 100 J/m². After BMT from Balb/c to CBA mice all recipients of unirradiated grafts died within 54 days. By contrast, after graft irradiation with 100 J/m² survival of recipient animals to day 80 was 59%. If these grafts were treated with 50 J/m² survival was only 28% with an increase in deaths due to GVHD. Hematopoiesis at day 80 in a group of survivors studied by Ig heavy chain allotyping indicated donor type hematopoiesis in 6 of 10 (50 J/m²) and 2 of 9 (100 J/m²). These data indicate that UV-B irradiation inhibits lymphocyte reactivity and can prevent GVHD. However, there is clear in vitro and in vivo evidence of stem cell damage, such that autologous marrow recovery was demonstrated in a proportion of recipients. In parent → F1 UV-irradiated transplants, sustained hematopoietic recovery was effected in the majority by donor stem cells.

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MATERIALS AND METHODS

Mice

The inbred strains used in the study were Balb/c (bred in our own colony), CBA (purchased from Harlan Olac, Bicester, Oxon, UK), and an F1 hybrid (Balb/c x CBA). All mice were 10 to 14 weeks old at the time of transplant and were maintained from day −2 and thereafter on sterile water incorporating gentamicin (2 mg/mL), sterile diet, and kept in pairs in autoclaved cages with sterile cage materials. In some experiments CBA recipients that have the Ig heavy chain allotype Igβ were chosen because this differs from Balb/c mice (Igβ).

UVR

UVR was performed in a purpose-built cabinet as described previously. Cells were placed in open Petri dishes and exposed to doses of UV-B varying from 0 to 300 J/m² using a battery of five linear fluorescent tubes (Phillips TL12; Phillips Lighting, Croydon, UK) emitting in the range 290 to 320 nm (peak 310 nm). Dosage
was calculated as follows:

\[ \text{J/m}^2 = \frac{\text{Intensity (uW/cm}^2) \times \text{time (seconds)}}{100} \]

**Viability**

Viability of splenic cells was assessed at various times after irradiation by means of trypan blue dye exclusion.

**MLC**

Spleens were removed aseptically and teased into RPMI 1640 (Imperial Laboratories, Andover, UK) supplemented with 2 mmol/L L-glutamine, 25 mmol/L HEPES buffer, penicillin 100 U/mL, streptomycin 0.1 mg/mL, and 10% fetal calf serum (FCS; GIBCO, Paisley, Renfrewshire, UK). The cells were passed several times through 25-g needles to obtain a single cell suspension and were washed twice in phosphate-buffered saline (PBS) at pH 7.2 and diluted to yield a final concentration of 2 \times 10^7/mL in supplemented RPMI. Reactivity in MLC was tested using cells derived from the Balb/c strain against similar cells from CBA animals. Stimulator cells were \( \gamma \)-irradiated (3,000 cGy) using a Cesium\(^{137} \) source, and the effect of UV-B irradiation on either stimulation or response was tested by irradiation with 20, 50, 100, or 250 J/m\(^2 \) as described above. Cells were tested in parallel MLCs as both stimulators and responders. Aliquots, 100 \( \mu \)L, of the appropriate responding and stimulating cell suspensions were dispensed in triplicate into 96-well microtiter trays (Cell-cult; Sterilin Limited, Feltham, UK). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO\(_2\). After 54 hours of incubation at 37°C in a humidified atmosphere the formation of precipitin lines was recorded using dark-field illumination.

**Transformation Assay**

Single cell suspensions were prepared as described above and were plated at a concentration of 2 \times 10^5/mL in triplicate wells of a 96-well round-bottomed microtiter plate and incubated at 37°C in a humidified atmosphere with 5% CO\(_2\). After 48 hours of incubation at 4°C in a moist atmosphere the formation of precipitin lines was recorded using a \( \beta \) scintillation counter (LKB-Wallac 1215, Milton-Keynes, UK).

**Granulocyte-Macrophage Colony-Forming Unit (CFU-GM) Assay**

CFU-GM assay was performed using a modification of the semisolid agar technique of Pike and Robinson.\(^9\) Nucleated bone marrow cells from Balb/c mice were plated at a density of 2 \times 10^6/mL over feeder layers consisting of 1 \times 10^6 leukocytes obtained from healthy human donors and shown previously to support the growth of murine marrow. The cells were either unirradiated or irradiated with 20, 50, and 100 J/m\(^2 \) of UV-B. The plates were incubated at 37°C in a humidified atmosphere with 5% CO\(_2\), and colonies consisting of greater than 40 cells were scored at day 14.

**Spleen Colony-Forming Unit (CFU-S) Assay**

CFU-S were assayed in both Balb/c and CBA mice as previously described.\(^9\) Briefly, 5 to 10 mice in each group were positioned in a perspex irradiation chamber and exposed to total body irradiation of 800 cGy from a linear accelerator (Philips Medical Systems, Crawley, UK) (100 Gy/min at 5 MeV under 1-cm perspex build-up in an O\(_2\)-flushed container at 135 cm source distance). Bone marrow nucleated cells (3 \times 10^6), either control or subjected to UV-B irradiation at doses of 50 and 100 J/m\(^2 \) were injected via the tail vein and spleen colonies were enumerated at day 12.

**Transplantation Procedure**

Recipient animals for BMT procedures were positioned in a perspex radiation chamber and exposed to a total of 800 cGy/min body irradiation at 100 cGy/min from a linear accelerator as described above. Radiation control animals received doses of 750, 800, 850, and 900 cGy. Donor marrow was harvested from the tibias and femurs of Balb/c or CBA animals by repeatedly flushing with culture medium through a 25-g needle. Donor spleen cells were prepared as described above and used as a single cell suspension. Aliquots of 0.2 mL RPMI containing 1.5 \times 10^5 nucleated cells (ratio 2:1; bone marrow: spleen) were injected via the tail vein into 10- to 14-week-old recipient animals. Appropriate irradiation and syngeneic controls (CBA) were studied. The effect of UV-B irradiation on the speed of engraftment was assessed in syngeneic Balb/c transplants by counting the number of nucleated cells in both femurs and tibias of recipient animals killed at days 7, 11, 15, and 21 after transplantation or by performing such counts on surviving allogeneic recipients observed for GVHD and killed at day 80. In these experiments groups of recipient animals were assessed daily for the presence of GVHD by monitoring weight, vitality, fur condition, alopecia, and soiling.

**Assessment of Chimerism Post-BMT**

Assessment was performed in two ways. Appropriate controls were included in all instances.

**Ouchterlony diffusion (for Ig heavy chain allotype).** This diffusion was performed in a group of BMT recipients of Balb/c marrow and splenocytes that received either 50 or 100 J/m\(^2 \) of UV-B radiation. Serum samples were obtained at day 80 and diluted 1 in 2 with PBS and pipetted into 3-mm round wells punched in 1.25% agarose (Miles Laboratories, Slough, UK). Murine polyclonal antiserum antibodies (anti-IgA; anti-IgB) reactive with Balb/c and CBA 101 sera, respectively, were not diluted. After 48 hours of incubation at 4°C in a moist atmosphere the formation of precipitin lines was recorded using dark-field illumination.

**Complement-mediated cytotoxicity.** This method was used for parent \( \rightarrow \) F1 procedures. Splenocytes (10³) from recipients killed at day 80 were tested against antisera to H-2\(^k\)-d and H-2\(^k\) (Cedarlane Laboratories, Hornby, Ontario, Canada). Two-stage incubations were performed: allo-antisera with cells for 60 minutes at 4°C followed by addition of rabbit complement (low-tox; Cedarlane Laboratories) for another 60 minutes at 37°C. Cellular viability was then assessed using ethidium bromide and acridine orange staining, and percent lysis was calculated. Cytolysis in nonchimeric animals was usually greater than 90% for H-2\(^k\)-d (Balb/c), greater than 90% for H-2\(^k\) (CBA), and greater than 90% using either antiserum (F1; CBA \( \times \) Balb/c). Controls also included spleen cells incubated with medium or complement alone.

**RESULTS**

**In Vitro Experiments**

Lymphocyte transformation in response to the mitogens PHA or Con A was reduced by a short exposure to UV-B of 20 J/m\(^2 \), which diminished the proliferative response of splenic lymphocytes to between 20% and 40% of control. At 100 J/m\(^2 \) of UV-B lymphocyte proliferation was abolished (Table 1).
There was a reduction in the proliferation of spleen cells in MLC after treatment with UV-B irradiation. The reactivity of Balb/c splenocytes in response to CBA stimulators was reduced to 13% of control when exposed to 20 J/m² and to less than 1% of control after exposure to 50, 100, and 250 J/m², respectively. Similarly, the response of CBA splenocytes to Balb/c stimulators was reduced to 26% of control when exposed to 20 J/m² and to less than 1% of control when treated with 50, 100, and 250 J/m² (Table 2). The ability of UV-irradiated cells to stimulate in MLC was well preserved at these doses by comparison (Table 2). Viability of spleen cells determined 24 and 72 hours after irradiation was reduced by 50 and 100 J/m² of UV-B (Table 3).

There was a progressive reduction in the recovery of CFU-GM progenitors after UV-B pretreatment to 23% and 9% of control with exposures of 50 and 100 J/m² respectively (Fig 1).

In Vivo Experiments

**Number of CFU-S.** The number of CFU-S obtained after irradiation of syngeneic marrow was reduced from a mean of 7 colonies/recipient animal to less than 1 colony/recipient animal (occasional CFU-S only seen) after irradiation of the marrow suspension with 50 or 100 J/m².

**Radiation controls.** A small proportion of animals survived after irradiation with 750 cGy (7%) and 800 cGy (4.5%). The remainder and all those receiving 850 or 900 cGy died in aplasia (Fig 2). At 800 cGy near-full thickness gut necrosis and hepatic necrosis were often seen at autopsy. For this reason a higher dose was not used in BMT procedures.

**Syngeneic BMT.** Twenty-eight of 31 CBA mice (90%) reconstituted with syngeneic cells survived to day 80 after which they were killed and shown to have normocellular marrow (nucleated cell count 25 to 45 × 10⁹) (Fig 2); the remainder died within the first 8 days during marrow aplasia.

**Parent → F1 BMT.** All recipients of non-UVR grafts died within 34 days with clinical features (hunched posture, weight loss, rough fur) indicative of GVHD (Fig 3). In some recipients postmortem examination was performed and confirmed the clinical diagnosis. When grafts were irradiated with 100 J/m² 76% of recipients survived to day 80; the remainder died early (< 14 days) in aplasia (15%) or later from GVHD (9%). At a higher dose survival was reduced to 25% (200 J/m²) and 0% (300 J/m²) with all deaths occurring with marrow aplasia, suggesting that increasing the dose of UVR was detrimental to stem cell engraftment (Fig 3).

**Balb/c → CBA BMT.** As in the previous group all recipients of unirradiated grafts died of GVHD, in this case within 54 days. Survival of animals grafted with marrow and spleen cells treated with 100 J/m² of UVR was 59%. In this group deaths occurring within 21 days were due to marrow aplasia (25%) and those occurring subsequently from GVHD (16%). A decrease in survival to 26% was seen with 50 J/m² UVR (Fig 4) since an increase in deaths associated with GVHD was seen (47%), with 27% dying as a result of aplasia.

**Reconstitution experiments.** The counts of femoral and tibial marrow taken from syngeneic Balb/c recipients on days 7, 11, 15, and 21 indicated that mice usually had full hematologic recovery by day 15 posttransplant. The kinetics of marrow reconstitution were retarded in syngeneic transplants after graft irradiation with 100 J/m² (Fig 5).

**Chimerism studies.** (1) Parent → F1 BMT: Fifteen recipients of grafts treated with 100 J/m² of UVR with normal marrow cellularity were assessed by cytotoxicity assay at day 80. Of these recipients, eight had donor-type,

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**Table 1. Influence of UVR on Response to Mitogens**

<table>
<thead>
<tr>
<th>Dosage of UVR</th>
<th>CBA PHA CPM ± SD</th>
<th>CBA PHA % Control</th>
<th>CBA Con A CPM ± SD</th>
<th>CBA Con A % Control</th>
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<tr>
<td>0</td>
<td>30,667 ± 6,265</td>
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<td>36,672 ± 7,275</td>
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<tr>
<td>20</td>
<td>6,064 ± 953</td>
<td>20</td>
<td>6,068 ± 1,894</td>
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</tr>
<tr>
<td>50</td>
<td>195 ± 46</td>
<td>&lt;1</td>
<td>204 ± 102</td>
<td>&lt;1</td>
</tr>
<tr>
<td>100</td>
<td>155 ± 37</td>
<td>&lt;1</td>
<td>164 ± 39</td>
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</tr>
<tr>
<td>250</td>
<td>114 ± 32</td>
<td>&lt;1</td>
<td>135 ± 16</td>
<td>&lt;1</td>
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</tbody>
</table>

n = 5.

**Table 2. Influence of UVR on MLC Reactivity**

<table>
<thead>
<tr>
<th>UV-L J/m²</th>
<th>Balb/c × UV CPM ± SD</th>
<th>Balb/c × UV % Control</th>
<th>CBA × UV CPM ± SD</th>
<th>CBA × UV % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29,677 ± 3,190</td>
<td>100</td>
<td>33,066 ± 3,946</td>
<td>100</td>
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<tr>
<td>20</td>
<td>24,657 ± 2,326</td>
<td>83</td>
<td>4,270 ± 614</td>
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</tr>
<tr>
<td>50</td>
<td>22,509 ± 1,010</td>
<td>76</td>
<td>137 ± 31</td>
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</tr>
<tr>
<td>100</td>
<td>23,283 ± 2,183</td>
<td>78</td>
<td>172 ± 88</td>
<td>&lt;1</td>
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<tr>
<td>250</td>
<td>21,604 ± 2,273</td>
<td>73</td>
<td>0 ± 0</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

n = 5.

Abbreviations: UV, irradiated with UVB; ×, γ irradiated.
Table 3. Viability of Murine Spleen Cells After UVR

<table>
<thead>
<tr>
<th>Time After Irradiation (h)</th>
<th>Dose of UV-B (J/m²)</th>
<th>0</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>85 (74-94)</td>
<td>61 (40-74)</td>
<td>52 (40-53)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>61 (52-76)</td>
<td>30 (12-66)</td>
<td>20 (16-38)</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean percentage viability (range); n = 3.

five mixed donor/recipient, and two recipient-type hematopoiesis. (2) Balb/c → CBA BMT: By contrast, only two of nine recipients of grafts irradiated with 100 J/m² of UVR were shown to have donor-type hematopoiesis, whereas one of nine showed mixed donor/recipient and six of nine had recipient-type hematopoiesis. With a lower dose (50 J/m²) of UVR fewer animals survived (vide supra), but a higher proportion (6 of 10) had donor-Ig allotype only demonstrable. Two showed mixed chimerism.

**DISCUSSION**

The aim of the present study was to explore the possibility of sustained engraftment of histoincompatible bone marrow without the use of postgraft immunosuppression. Despite the successful reduction in acute GVHD observed when donor marrow is depleted of mature T cells, there is now clear evidence that the incidence of rejection increases, which may depend in part on the degree of major histocompatibility complex (MHC) disparity between donor and recipient. There are now several reports of the use of UVR to prevent HVG responses. There is uniform rejection of canine bone marrow allografts when recipient animals are transfused with blood from the donor before transplant; prior UV treatment of donor blood has been shown to prevent this. Similarly, the survival of histoincompatible pancreatic islets and cardiac allografts in rats is prolonged when donor-specific blood transfusions treated with UVR are given. The inactivation of specialized class II bearing cells in transfused blood appears to be central to this tolerizing effect. When recipients of murine pancreatic islet allografts are pretreated with antiserum to Ia antigens prolonged allograft survival is observed. If the abrogation of reactivity in MLC observed after appropriate doses of UVR accurately predicts in vivo allogeneic responses then both GVHD and HVG/rejection could be abolished. In a murine popliteal lymph node assay in vivo GVH and HVG responses were eliminated after irradiation with a dose of UVR twice that required to abolish in vitro MLC responses.

For UV radiation to be successfully used in clinical transplantation it is essential that alloreactive responses are abolished, whilst the function of specialized cells such as...
hematopoietic stem cells and pancreatic islets are retained. Lau et al. reported prolonged survival and function of the latter when irradiated with 900 J/m² of UV-B (mean wavelength 310 nm) and transplanted from Lewis rats into diabetic ACI recipients. In vivo, GVH reactivity can also be reduced or abolished. In canine recipients of autologous bone marrow administered postgraft transfusions of histoincompatible leukocytes, GVHD was prevented by UV-C irradiation at a dose of 1,000 mJ/cm² but not at a lower dose of 20 mJ/cm². Acute GVHD developed in 100% of recipients of unirradiated transfusions. The same investigators had previously shown that UV-B but not UV-C irradiation allowed adequate preservation of stem cell function whilst proliferative responses to PHA were abolished.

We have sought to establish that a similar window of efficacy exists in a murine bone marrow transplant model so that the possibility of irradiating both bone marrow and splenocytes before transplantation might be explored. However, a dose of 50 J/m² was required to reduce proliferation to mitogens and response in MLC to less than 1%; this dosage reduced proliferation of CFU-GM progenitors to 23% of control. At a dose of 100 J/m² clonogenic and mitogenic responses and responder function in MLC were reduced to less than 1%. At the same time ability to stimulate in MLC was relatively well preserved. Our results indicate that spleen cell viability is compromised by 50 to 100 J/m² of UV-B, and this might partly account for the differential effect on MLC responses. Cahill et al. have shown that CFU-S recovery in mice is retained after 50 to 100 J/m² of UV-B radiation, whereas response and stimulation in MLC is abolished by 50 J/m². However, in our studies, whilst CFU-S are not completely eliminated, their growth is sharply reduced by 50 or 100 J/m² of UV-B (doses required to suppress PHA proliferation). The UV-I. source used in our studies emits maximal energy at 310 nm compared with 302 nm in the work of Cahill et al. It may be that the differences in our observations could be partially explained by these variations. In addition, our results indicate that hematopoietic reconstitution is retarded in syngeneic experiments when bone marrow and spleen cells receive 100 J/m² of UVR before injection.

Our in vivo results confirm that BMT from Balb/c → CBA or Balb/c → F1 (Balb/c × CBA) using unirradiated marrow/spleen cells is uniformly lethal due to GVHD. A dose of 800 cGy was established as most suitable for these procedures because less than 5% of unreconstituted animals survived whereas 90% of CBA recipients of syngeneic grafts became long-term survivors. BMT using 100 J/m² of UVR resulted in survival to day 80 in the majority of cases. The higher doses used in parent → F1 BMT procedures were detrimental to survival, whilst the dose of 50 J/m² tested in Balb/c → CBA transplants reduced survival, in part due to an increase in GVHD.

However, not all survivors of BMT using 100 J/m² UVR to the graft showed donor-type hematopoiesis. In parent → F1 BMT the majority of recipients had donor (53%) or mixed donor/recipient (33%) hematopoiesis, indicating that while donor engraftment predominated autologous recovery could also occur, in some cases alongside donor hematopoiesis. In two animals the graft did not take and autologous recovery permitted survival to day 80. By contrast in Balb/c → CBA transplants most animals showed recipient type hematopoiesis, although it is of interest that donor-only hematopoiesis became established in two of nine animals studied. This difference is probably because in parent → F1 experiments the recipients are unable to reject parental grafts whereas this is not so in Balb/c → CBA transplants, and in MLC stimulation was relatively well preserved between the two strains. However, despite evidence of in vitro stem cell damage, UV-irradiated grafts can re-establish hematopoiesis to day 80 with normocellular
bone marrow and absent clinical GVHD in some recipients. By contrast, Pepino et al showed in rats that all recipients of grafts irradiated with 900 J/m² became long-term survivors with donor-type hematopoiesis. Both strain and species differences may be important when considering these differences.

UVL has numerous effects on alloreactivity that include impaired antigen presentation and interleukin (IL) production, as well as a failure to generate intracellular calcium fluxes after appropriate stimulation.30 The formation of clusters of lymphocytes with dendritic cells in response to mitogens is reduced by UVR.32 These data and those from transfusion studies (vide supra) suggest that inactivation of donor antigen-presenting cells readily occurs after UVR. However, these observations do not explain the inability of responding T lymphocytes in donor bone marrow and splenocyte populations to mediate GVH reactions when UV irradiated. Nonetheless, the ability to prevent GVHD is consistent with abolition of responder function in MLC. In addition to initiating GVH responses, donor T lymphocytes produce cytokines that may promote engraftment.33 If such T cells are inactivated by UV-L then this might not occur and engraftment could be retarded. However, a recent report indicated that expression of the IL-1 gene in cultured human keratinocytes was upregulated after irradiation with UV-B, (290 to 320 nm) as shown by enhanced expression of mRNA.34 It is not known whether the expression of other sequences encoding for hematopoietic growth factors produced by T lymphocytes is altered after UVR. Treatment with UV-L reduces lymphocyte viability and it is likely that this effect would predominate over any tendency toward enhanced cytokine production.

In conclusion, UV-B irradiation has a damaging effect on murine stem cells such that hematopoietic reconstitution after transplantation is slowed resulting in deaths during marrow aplasia. Coincidentally, alloreactive responses are reduced such that a suitable dose allows for survival without GVHD of a significant proportion of recipient animals across a major histocompatibility barrier. However, autologous marrow recovery with donor graft failure or rejection and mixed allologenic chimerism are also seen, particularly in CBA recipients of Balb/c grafts, probably as a result of damage to donor stem cells and relatively well-preserved host responses as evidenced by the findings in MLC. In humans published data suggest that UV-L might be used at a dose that spares stem cells in clinical transplantation.

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REFERENCES

5. Gale RP, Bortin MM, Van Bekkum DW, Biggs JC, Dicke KA, Gluckman E, Good RA, Hoffmann RG, Kay HEM, Kersey JH, Marmont A, Masaoka R, Rimm AA, Van Rood JJ, Storb R: Treatment with UV-L reduces lymphocyte viability and it is likely that this effect would predominate over any tendency toward enhanced cytokine production.
19. Lord BI, Schofield R: Haemopoietic spleen colony-forming


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