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THE EFFECT OF SKIN HOMOGRAFT REJECTION ON
RECIPIENT AND DONOR MIXED
LEUKOCYTE CULTURES

By JOOST J. OPPENHEIM,* M.D., JACQUELINE WHANG, M.D., AND
EMIL FREI, 3RD, M.D.

(From the National Institutes of Health, National Cancer Institute,
Medicine Branch, Bethesda, Maryland)

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The discovery of the mitogenic (1) and lymphocyte transforming (2) properties of phytohemagglutinin (PHA) was soon followed by the finding that many antigens (3, 4) and vaccines (5) were also able to stimulate peripheral lymphocytes to proliferate in short-term cultures, provided the cell donor had been previously sensitized with the antigen. Histocompatibility antigens also produce lymphocyte transformation and mitoses without preceding sensitization in mixed leukocyte cultures of unrelated individuals (6). As the genetic relationship increases from unrelated subjects to siblings, and thence to identical twins, the lymphocyte proliferation of their mixed leukocyte cultures diminishes (7). The degree of lymphocyte transformation in mixed leukocyte cultures has also been reported to be proportional to the degree of cross-reactivity of graft rejection of the subjects' skin by a third unrelated recipient (8). To define further the relationship between lymphocyte transformation and homograft rejection we have investigated the changes in the reaction of mixed leukocyte cultures of graft recipients and a donor temporally before, during, and after first and second skin transplants (9).

Methods

The 3 subjects were two female graft recipients, "A" and "B" aged 44 and 49 years respectively, and a 61-year-old male graft donor, "C." Patient A had an exacerbation of her recurrent keratoacanthoma, but was free of any systemic symptoms except for a transient episode of iron deficiency anemia during the latter part of the study after the second skin grafting. Both patients B and C had a history of malignant melanoma, but were asymptomatic and had been free of any recurrent disease for more than 5 years. The subjects had not received immune suppressant therapy for more than a year prior to the study. All 3 had normal gamma globulin levels as determined by serum protein electrophoresis and normal febrile agglutination titers to the *Salmonellae* groups. Subjects B and C were tuberculin (PPD intermediate strength)¹ positive, but A was not.

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¹ PPD is purified protein derivative, Merck, Sharpe, and Dohme, West Point, Pennsylvania.

Two 8 mm full thickness normal skin punch biopsy specimens were transplanted from subject C to A and B each for the first time on day 0 of the study, and once again 118 days later. Both after the first and second transplants the skin grafts were examined frequently until totally rejected.

On 23 occasions over a 6-month-period before, during, and after grafting 35 to 50 ml of the subjects' blood was drawn by venipuncture and heparinized with 10 μ g/ml. The subjects' white blood cell (WBC) count and differential were determined. The red blood cells were sedimented at room temperature for 1 to 2 hours at a 15° angle from the horizontal and the resulting WBC rich plasma aspirated. This was then added to sufficient minimal essential medium² (supplemented with 50 u. penicillin, 50 μ g streptomycin, and 0.1 mM glutamine/ml) to obtain a final leukocyte concentration of 1000 to 2000/ml³ and an autologous plasma concentration of 30 per cent. Thereafter, equal volumes of the cell suspensions from the graft donor and recipient pairs were mixed in the following combinations: both recipients A and B, recipients and donor A and C, and B and C, and all three together A, B, and C. Twelve ml aliquots of these peripheral leukocyte mixtures were incubated in stationary sealed bottles at 37°C for 5 days. Beginning on the 9th day after the first graft the mixed cell suspensions were cultured in duplicate. 40 Per cent of this second set of cultures was harvested on the 3rd day. The remaining cell suspension was then replenished with an equal volume of cell-free medium containing 30 per cent of the subjects' plasma, and this was totally harvested on the 7th day. Duplicate cultures that were incubated for 7 days without replenishment of their medium generally supported less cell proliferation than those treated as above. The cell mixtures were cultured on 3 occasions prior to grafting and retested 20 times thereafter (see Fig. 1 for the precise test schedule after grafting). On 10 occasions, before, during, and after each graft rejection, unmixed WBC both from the donor and the recipients were cultured in autologous plasma. On the 13th and 28th days after the 2nd grafts, during and after the height of the second set homograft reaction, the donor cells were cultured in each of the recipient's plasma, and the recipients' cells in the donor's plasma. The subject's cells were cultured with PHA and/or antigens about 6 times during the study to check the status of their lymphocyte response. On 4 occasions, before and after second grafting, duplicate sets of mixed and unmixed cell suspensions were washed twice with isotonic saline and cultured in medium with 30 per cent fetal calf serum.³ Their cell growth was compared with that of identically treated cultures grown in human plasma.

0.20 ml of 1:100 diluted polystyrene latex particles⁴ were added 4 hours prior to harvesting to those cultures incubated for 5 and 7 days. The WBC count of the cell suspensions was determined and they were then harvested by 500 RPM centrifugation at room temperature for 5 minutes (International centrifuge with No. 240 head). The cell button was then slowly resuspended and fixed with modified Carnoy's solution (8:1 absolute methyl alcohol: glacial acetic acid) for 10 minutes. The cells were recentrifuged, resuspended in $\frac{1}{2}$ to 2 cc of fresh fixative, pipetted onto alcohol cleaned slides, air dried, and stained with Giemsa (Harleco).⁵

The slides were analyzed independently as unknowns by 2 investigators for mitotic index (No. of mitoses per 1000 mononuclear cells) and the proportions of small lymphocytes, macrophages, and transformed lymphocytes. The phagocytic macrophages could often be identified by their uptake of polystyrene particles, eosinophilic granular cytoplasm, irregular shape, and their homogeneous nuclear chromatin. In contrast, the non-phagocytic transformed lymphocytes had basophilic non-granular cytoplasm and prominent nucleoli. The small lymphocytes had scanty pale blue non-granular cytoplasm and pyknotic nuclear chromatin.

² Eagles modified Spinner medium, Flow Labs, Rockville, Maryland.

³ Microbiologic Associates, Bethesda, Maryland.

⁴ Polystyrene latex particles (1.3 μ diameter) Dow Chemical Co., Midlands, Michigan.

⁵ Harleco—Arthur H. Thomas Co., Philadelphia, Pennsylvania.

The mitotic indices of the cultures were low, and generally manifested the same changes as did the lymphocyte transformation and these 2 parameters were therefore reported together in the results.

On 8 occasions 1 to 4 μ c of tritiated thymidine (TdRH^3)⁶ was added per ml of cell suspension in single or divided doses from 4 hours to 4 days prior to harvesting. Radioautographs were prepared with Kodak AT 10 stripping film (10), and were exposed for 1 to 2 weeks. They were then analyzed for the proportions of labeled mononuclear cells.

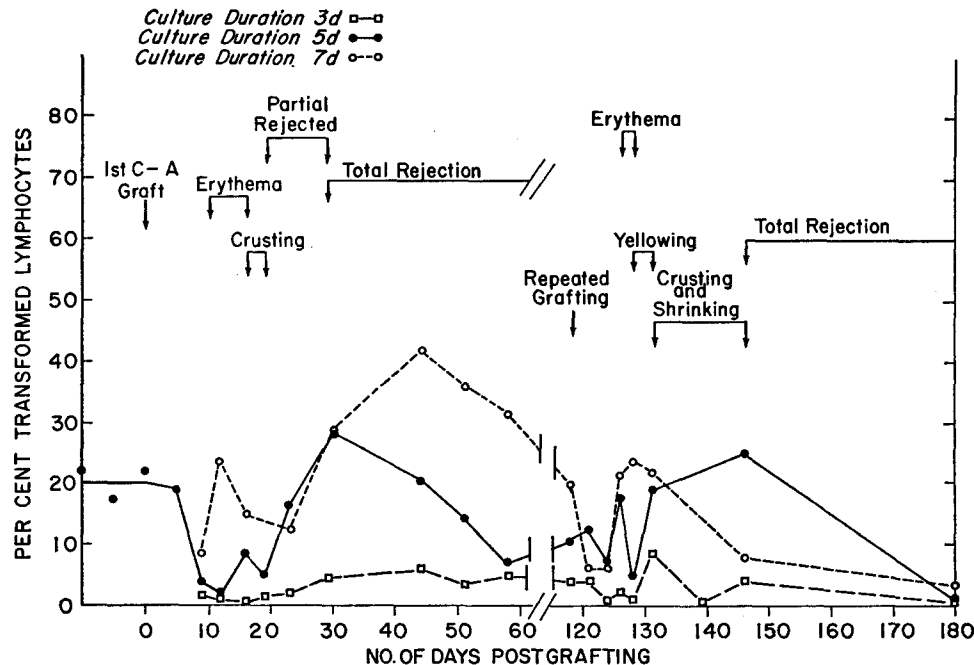


FIG. 1. The relationship of the graft status of recipient A to the lymphocyte transformation in mixed leukocyte cultures of her cells with those of graft donor C.

Concomitantly, on the 51st and 58th day after the 1st graft, 5 ml aliquots from 28 different cell suspensions treated with different doses of TdRH^3 for varied periods of time were analyzed for total TdRH^3 uptake. The aliquots were centrifuged at 1000 RPM for 10 minutes, the resulting cell button washed 3 times with ice cold isotonic saline, and dried at 100°C for 1 hour. The cell button was hydrolyzed with 0.5 ml of 5 per cent sodium hydroxide at 60°C for 30 minutes. 0.2 ml of the hydrolysate was added to 18 ml of Turner's solution (7 per cent toluene, 30 per cent methanol, 0.3 gm per cent PPO, and 5 mgm per cent POPOP). The solution was cooled to 4°C, and the counts per minute determined in a Tricarb liquid scintillation counter.⁷

Chromosome preparations of the mixed leukocyte cultures incubated for 5 or 6 days were made by a modification of the method of Moorhead *et al.* (11). The cells were exposed to

⁶ Schwarz BioResearch Inc., Orangeburg, New York.

⁷ Packard Instrument Co. Inc., La Grange, Illinois.

0.2 μ /ml colcemide⁸ for 2 hours prior to harvesting. Thereafter they were treated with 1 per cent hypotonic sodium citrate at room temperature for 30 minutes, fixed with a 1:1 mixture of absolute ethyl alcohol and glacial acetic acid for 20 minutes, and spread by a rapid air-drying technique. The ratio of XX to XY karyotypes was determined by analyzing up to 60 metaphases per male-female leukocyte mixture. The male graft donor and female graft recipients' mixtures each were thus analyzed before each graft, and on the 30th and 28th day after receiving the 1st and 2nd graft respectively. The female:male ratio of metaphases in the leukocyte mixtures of 4 pairs of unrelated ungrafted volunteers was similarly determined.

RESULTS

Unmixed, unstimulated WBC cultures of the graft recipients and donor whether grown in autologous or each other's plasma or in calf serum, manifested only 0 per cent (0 to 1.5) and 0.2 per cent (0 to 5.1) lymphocyte transformation respectively. Their mitotic response was less than 1 per 2000. The proportions of mononuclear cells labeling with TdRH³ in these cultures were only 0 per cent (0 to 0.5) in human plasma, and 0.4 per cent (0 to 5.0) in calf serum. The ability of the subjects' lymphocytes to respond to PHA and antigens such as purified protein derivative and vaccinia vaccine remained normal throughout the study. The duplicate mixed as well as unmixed unstimulated leukocyte cultures that were incubated in calf serum, rather than human plasma, usually manifested slightly more lymphocyte transformation and TdRH³ uptake. This was probably due to a low grade cellular response to the heterologous antigens present in the calf serum.

Only the results of those mixed cultures incubated for 5 days will be discussed in detail because they are the only available determinations for the pregraft period, and because they parallel the response of the cultures incubated for 3 and 7 days. Figs. 1 through 6 provide all the data for each of the culture durations. The proportion of transformed lymphocytes generally increased with increased duration of incubation. The 3 day cultures were least and the 7 day cultures most sensitive to the effects of skin grafting on the lymphocyte transformation in the mixed WBC cultures. However, the 7 day cultures manifested greater variability of the response than those incubated for only 5 days, possibly because they develop more nutrient deficiencies. Some of the progeny of transformed lymphocytes in 7 days become morphologically indistinguishable from small lymphocytes, but can be identified as such by the fact that they label with TdRH³. Both these factors could at times result in falsely low determinations of lymphocyte transformation in the 7 day cultures.

The median lymphocyte transformation prior to grafting of leukocyte mixtures of graft donor C and recipient A was 20 ± 2.8 per cent, and that of C and B was 15 ± 3.4 per cent (Figs. 1 and 2). Perigraft erythema appeared in recipients A and B, 12 and 19 days respectively after grafting. The grafts became encrusted and partially rejected in 16 and 23 days, and were totally rejected by

⁸ Ciba Pharmaceutical Products Inc., Summit, New Jersey.

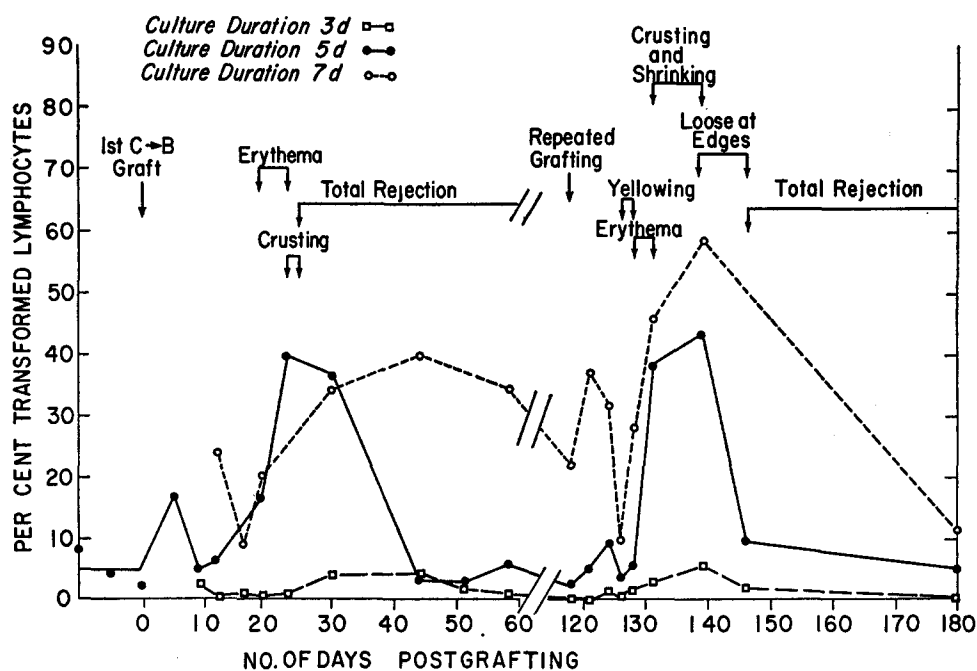


FIG. 2. The relationship of the graft status of recipient B to the lymphocyte transformation in mixed leukocyte cultures of her cells with those of graft donor C.

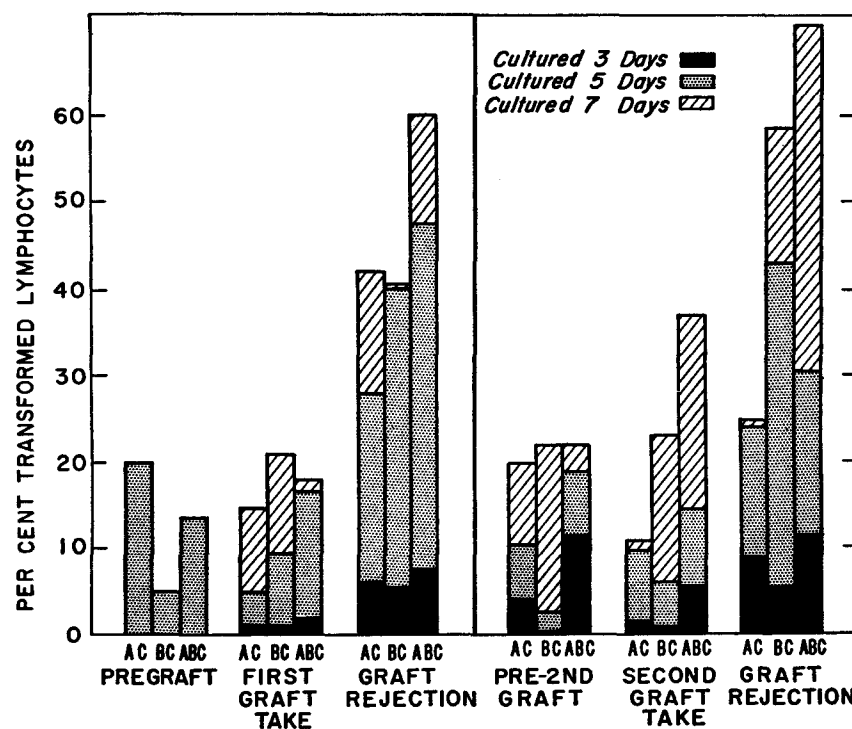


FIG. 3. A comparison of the median pregraft lymphocyte transformation with the median pregraft rejection and maximum graft rejection response in mixed leukocyte cultures of graft recipients and donor pairs AC, and BC and all 3 together (ABC).

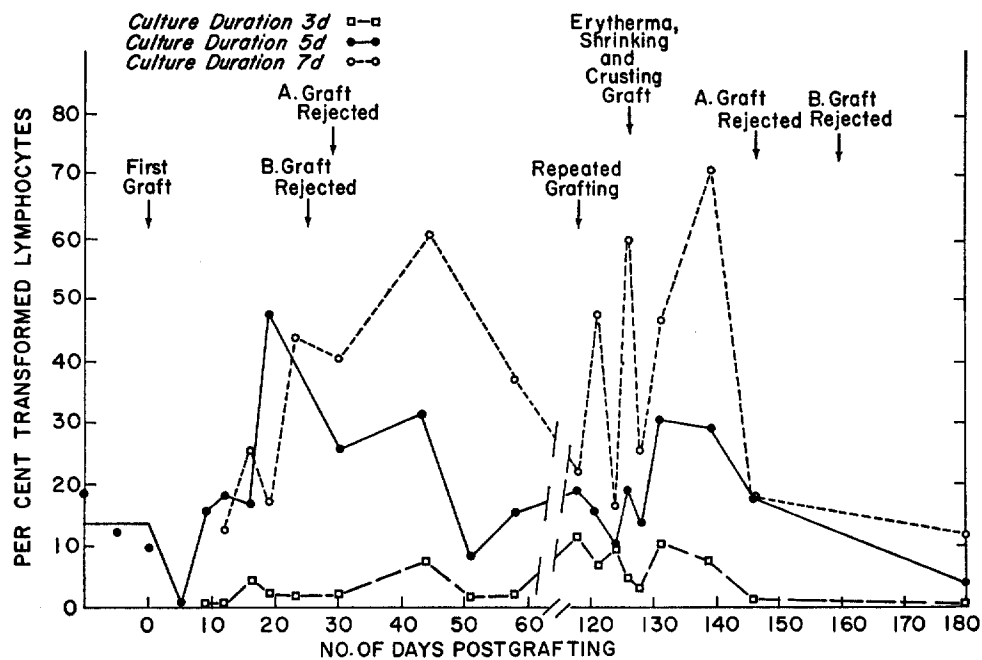


FIG. 4. The relationship of graft status to the lymphocyte transformation in mixed leukocyte cultures of both graft recipients' cells, A + B, with those of the donor C.

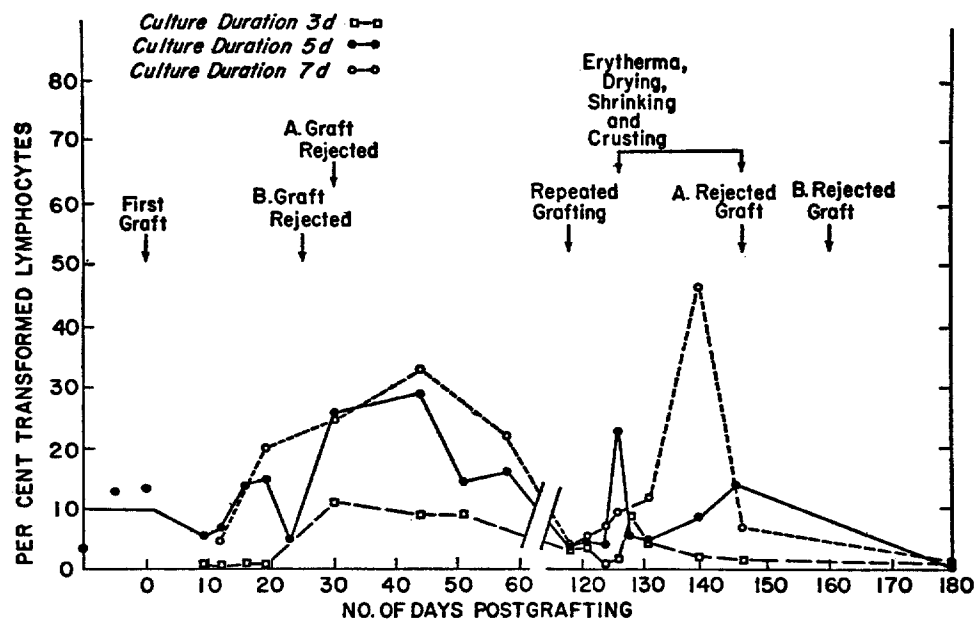


FIG. 5. The relationship of graft status to the lymphocyte transformation in mixed leukocyte cultures of both the graft recipients A and B.

both recipients by the 25th day after grafting. There was marked increase in the lymphocyte transformation of the A and C leukocyte mixture to a maximum of 28 per cent by the 30th day, and of B and C to 41.5 per cent by the 23rd day after grafting. This enhanced response was transient and returned to pregraft levels within 3 weeks. On day 118, just prior to second skin grafting, the proportions of transforming lymphocytes in the leukocyte mixtures of A with C, and

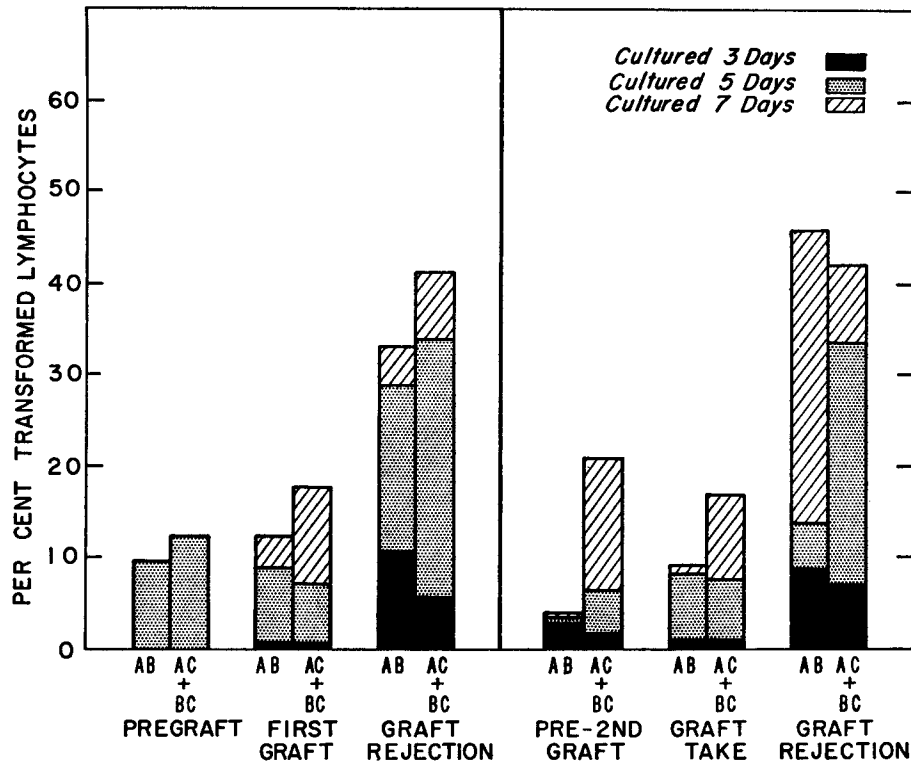


FIG. 6. A comparison of the average donor-recipient mixed leukocyte culture lymphocyte transformation with that of the recipient-recipient cell mixtures before and during first and second skin homograft reactions.

B with C were 10.7 and 2.3 per cent respectively. Six days later the grafts had taken and appeared viable in both recipients. This time the recipient perigraft erythema appeared sooner at 8 to 10 days, and the grafts were encrusted and partially rejected in 10 to 13 days. Thereafter they appeared non-viable, but the crusts did not fall off until 26 days after the 2nd graft. The lymphocyte response increased to 14.5 per cent in the A and C mixture, and to 38.5 per cent in the case of B and C by the 13th day after the second graft. This rise persisted for less than 2 weeks and returned to pregraft levels of 1 and 5 per cent respectively by the end of the study.

The proportion of transformed lymphocytes present in the combined mixtures of both recipients with the donors' leukocytes (A, B, and C) exceeded the response in cultures containing only 2 cell populations on 90 per cent of the occasions during the study (Fig. 3). The pregraft response of the leukocyte mixtures of A, B, and C was 13.4 ± 4.9 per cent (Fig. 4). This rose to 47.7 per cent by day 19, during the first graft rejection, but diminished within 4 weeks. Just prior to the second graft on day 118 the response was 19 per cent. It increased to a maximum of 30.8 per cent within 13 days at the time of the second set rejection but had again decreased in 2 weeks and was back down to 3.7 per cent by day 180.

TABLE I
Percentage of Mononuclears Labeled with Tritiated Thymidine

The proportion of mononuclear cells labeling with tritiated thymidine before and after grafting in donor-recipient AC, BC, and ABC, and recipient-recipient (AB) mixed leukocyte cultures.

| Mixture | Pregraft control | Day 23 | Day 51 | Day 58 | Pre- 2nd graft | Day 8 | Day 21 | Day 64 |
|---------|------------------|--------|--------|--------|----------------|-------|--------|--------|
| AB | 2.0 | 5.0 | 5.0 | 7.0 | 3.0 | 16.5 | 3.6 | — |
| AC | 16.0 | 26.0 | 11.5 | 3.5 | 8.0 | 14.4 | — | 0.3 |
| BC | 1.6 | 25.0 | 2.0 | 4.0 | 1.0 | 2.6 | 20. | 3.0 |
| ABC | — | 18.0 | 6.5 | 7.0 | 17.2 | 14.5 | 22. | 2.4 |

The leukocyte mixture of the 2 recipients A and B manifested 10.1 ± 5.6 per cent lymphocyte transformation before grafting (Fig. 5). Thirty days after the 1st grafts this increased markedly but only temporarily to 26 per cent. Prior to the second grafting this had decreased to 3.9 per cent. Eight days subsequently the lymphocyte transformation was transiently enhanced to 23.2 per cent, but it had again decreased within 3 weeks. The average increase in the response of the graft recipient-donor leukocyte mixtures was not significantly different from that of the recipient-recipient cultures (Fig. 6).

The degree of cytotoxicity as manifested by the appearance of damaged, pyknotic, and fragmented cells varied during the study, but was subjectively somewhat more evident prior to the appearance of signs of graft rejection. The changes in the pre- to postincubation WBC counts of the cultures were inconsistent and variable. They failed to provide a quantitative measure of cell proliferation presumably because of the development of marked leucoagglutination and the variable cytotoxicity.

The proportion of mononuclear cells labeling with TdRH³ on radioautographs increased temporarily at the times of first and second graft rejection concomitant with the increase in transforming lymphocytes (Table I). This was clearly evident in the recipient-recipient as well as donor-recipient mixtures 23 days

after the first graft. Because of increased cytotoxicity in some of the cultures 2 of the cultures show the increase 8 days after the second graft, and the others not until the 21st day.

The extent of desoxyribose nucleic acid labeling with TdRH³ as measured by the scintillation counter agrees well with the proportions of labeled mononuclears on radioautographs (7). Both these parameters of response generally agree

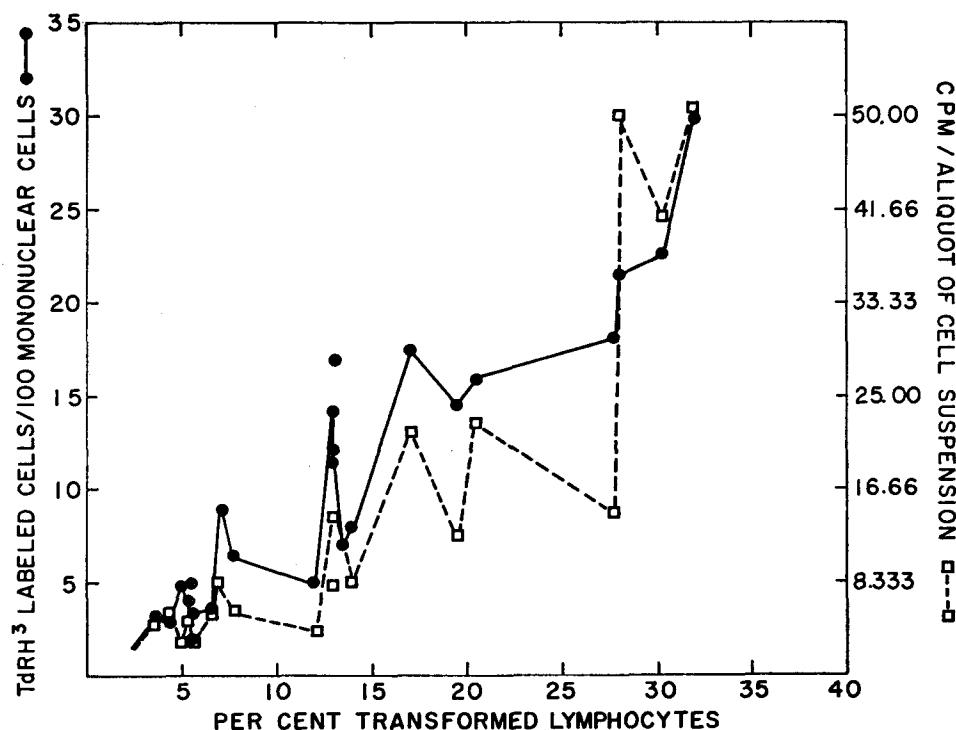


FIG. 7. The proportion of mononuclear cells labeling with TdRH³ on radioautographs (●—●) is compared with the total counts per minute of isotope uptake (□—□) of the mixed leukocyte cultures ranked in order of increasing lymphocyte transformation.

with our analysis of the degree lymphocyte proliferation using only morphologic criteria (Fig. 7). The agreement between CPM and proportions of cell labeling while somewhat variable, agreed still better with one another than they did with lymphocyte transformation. The median proportion of morphologically transformed lymphocytes that labeled with TdRH³ was 80 per cent with a standard deviation of ± 19.8 per cent. Eight cultures treated with 1 to 4 μ C TdRH³/ml in 4 divided doses over a 96-hour-period, all manifested considerably less lymphocyte transformation and less isotope uptake than did duplicate cultures exposed to 1 to 4 μ C TdRH³/ml for only 16 hours prior to harvesting. This is

presumably due to the cumulative injurious effects of prolonged intracellular irradiation of the cells exposed from the 2nd day of incubation.

Only those mixed leukocyte cultures that were incubated for 5 days and exposed to the mitotic arresting effect of colcemide, accumulated sufficient mitotic figures to manifest significant changes in the mitotic index. Significant increases over the pregraft levels occurred in all the mixtures by the 30th day after the 1st graft (Table II). The pregraft mitotic indices of cultures that were treated similarly but incubated for 6 rather than 5 days prior to the 2nd grafts were higher. Repeated determinations on the 28th day after the 2nd grafts failed to manifest any increase over these higher pregraft mitotic indices. This was at a time when the peak post-2nd graft lymphocyte transformation had already

TABLE II
Chromosome Preparations of Mixed WBC Cultures

The changes in mitotic index and ratio of XX to XY metaphases in chromosome preparations of graft recipients and donor mixed leukocyte cultures before and after grafting.

| Culture duration..... | Mixture | 5 days | | 6 days |
|-------------------------------|---------|----------|------------------------|--------------------------|
| Graft status..... | | Pregraft | Day 30 after 1st graft | Pre- 2nd graft (day 118) |
| Mitotic index | AB | 2 | 9 | 7 |
| | AC | 10 | 45 | 20 |
| | BC | 0 | 16 | 20 |
| | ABC | 2 | 21 | 53 |
| Recipient (♀)/donor (♂) ratio | AC | 3:2 | 5:1 | 2:1 |
| | BC | 1:1 | 5:1 | 4:1 |

been passed, in contrast to the maximal lymphocyte proliferation still manifest on the 30th day after the 1st graft.

The ratio of male to female metaphases, as determined from the XY *versus* the XX karyotypes, was approximately even in the mixed leukocyte cultures of 4 pairs of unrelated ungrafted volunteers, and in the cultures of A and C, and B and C prior to grafting (Table II). Thirty days after the 1st grafts this ratio changed temporarily to greater than 5:1 female graft recipient metaphases to every male graft donor mitosis in the cultures of A + C, and B and C. The recipient to donor ratio of metaphases was still uneven prior to the 2nd grafts but had returned to 1:1 on the 28th day after the 2nd grafts in the cultures of A and C. The preparations of B and C lacked sufficient numbers of analyzable metaphases and manifested excessive cytotoxicity at this time. The chromosome studies done 28 days after the 2nd grafts may have failed to manifest the increased graft recipient (female) leukocyte proliferation because by this time the maximal lymphocyte transformation had already subsided.

DISCUSSION

Mixed cultures of the leukocytes from female skin graft recipients with those of a male skin donor manifested a temporarily enhanced lymphocyte proliferation just after the first and second set graft rejections. Concomitant temporary increases were noted in the mitotic indices of the cultures after the first graft and in the proportion of mononuclear cells labeling with TdRH³ both after the first and second grafts.

The change from an even ratio of female:male metaphases in the pregraft leukocyte mixtures to greater than 5 graft recipient for every donor metaphase occurred during the period of enhanced lymphocyte transformation after the first graft. Since there was no discernible increase in cytotoxicity during graft rejection, a selective decrease in the proportion of donor cell divisions is not a likely cause of this marked change. It is therefore probably due to a temporary increase in the numbers of "sensitized" cells in the peripheral circulation of the graft recipient that are stimulated to transform in the presence of leukocytes from the skin graft donor. Thus donor leukocytes can stimulate increased recipient lymphocyte transformation after the recipient develops an immune reaction to donor skin. Conversely, the *in vivo* administration of donor leukocytes results in accelerated skin graft rejection (12). This indicates that skin and WBC have some histocompatibility determinants in common and suggests that the appearance of the increased numbers of recipient transformed lymphocytes in the presence of donor leukocytes after donor skin rejection is a reflection of that homograft reaction.

The changes in lymphocyte transformation in the mixed leukocyte cultures of the 2 graft recipients responded qualitatively and quantitatively in a fashion similar to those of the donor and recipients during graft rejection. Their enhanced lymphocyte proliferation could presumably be due to the presence of stimulating histocompatibility differences that the 2 recipients have in common with the donor-recipient pairs. This explanation is supported by the findings of a high degree of cross-reactivity of *in vivo* homograft rejection in a heterogeneous human population (13).

The *in vivo* second set skin graft rejection occurred considerably sooner after grafting, and the reaction was of shorter duration than the rejection of the 1st grafts. In all the mixed leukocyte culture combinations the heightened lymphocyte proliferation also appeared earlier after the second grafts, persisted for a shorter period of time, but was quantitatively similar to the response after rejection of the first graft. Thus changes in the rate of *in vivo* graft rejection were reflected by corresponding changes in the timing of the enhanced *in vitro* lymphocyte proliferation. This supports the suggestion that serial studies of donor-recipient mixed leukocyte cultures can detect the onset of graft rejection (14). Patients receiving *in vivo* immune suppressant chemotherapy develop marked inhibition of their *in vitro* PHA and antigen stimulated lymphocyte transforma-

tion while on therapy (15). Thus the inhibition of the mixed graft donor-recipient cultures may also prove helpful in determining the dose and duration of immune suppressant therapy needed to prevent graft rejection.

Rabbit antihuman WBC sera are mitogenic and transform human lymphocytes in culture (16). We were unable to demonstrate the appearance of an analogous recipient antidonor WBC humoral factor. Leukocytes from the donor and the recipients failed to proliferate when grown by themselves either in autologous or in each others plasma, or in calf serum even at the time of maximal lymphocyte transformation after the second set rejection. Furthermore, if the enhanced lymphocyte transformation was mediated by a recipient humoral factor, the number of donor metaphases should have increased rather than those of the recipients. Mixed cultures of different ungrafted rabbits' spleen cells are also stimulated to proliferate as indicated by their increased TdRH³ uptake. This response is inhibited by separating the spleen cell populations with a millipore filter. However, this does not inhibit the ability of PPD placed on one side of such a partition to stimulate cells on the other side (17). These findings favor the view that cell to cell contact is necessary for the mixed leukocyte reaction rather than mediation by a humoral factor. This may be the *in vitro* reflection of the inability to passively transfer normal homograft rejection by administering "hyperimmune" sera (18), whereas peripheral WBC (12), and leukocyte extracts can do so readily (19).

In rabbits the numbers of hypertrophied pyroninophilic cells in the regional lymph node draining a homograft site become markedly diminished on the 4th day of graft rejection (20). This finding is the basis for the suggestion that the lymphocyte is a "messenger" of immune information which redistributes to remote nodes with the information that originated in the regional node (21). We would like to speculate that the demonstrated temporary increase in the number of "sensitized" cells in the graft recipients' peripheral circulation after graft rejection is a manifestation of such a lymphocyte redistribution from the regional to systemic nodes. This is the most likely explanation of the short duration of the enhanced lymphocyte proliferation *in vitro* compared to the prolonged persistence of the *in vivo* second set graft rejection phenomenon (22). We have attempted to duplicate the postgraft enhanced lymphocyte proliferation by hyperimmunizing 4 volunteers with an antigen. Even though they were given the *Salmonella typhosa* endotoxin daily for 2 weeks, there was no significant change in their *in vitro* lymphocyte transformation with the antigen. Perhaps this is because it was administered intravenously, and therefore did not manifest any easily detectible lymphocyte redistribution (23).

The findings indicate that lymphocyte transformation is a significant indicator of the homograft reaction. It may be a more relevant guide to the study of histocompatibility than hemagglutinins (24), leukoagglutinins (25), hemolysis (26), and cytotoxicity (27). Unlike them it does not involve a humoral fac-

tor, but an intercellular reaction and thus simulates the conditions of *in vivo* homograft rejection more closely.

SUMMARY

The lymphocyte proliferation in repeatedly studied mixed leukocyte cultures of peripheral white blood cells from a skin graft donor and 2 recipients was significantly increased at the time of graft rejection. This was determined from the increased proportions of mononuclear cells labeling with tritiated thymidine, increased mitotic indices, and the appearance of increased numbers of transformed lymphocytes after rejection of 1st and 2nd skin grafts. The temporarily enhanced response occurred sooner and was of shorter duration after the second than after the first graft, but was quantitatively similar each time. The cell proliferation in the mixed leukocyte cultures of the two recipients was similarly affected by the homograft rejections. The cultures containing three cell populations usually manifested a greater lymphocyte response than corresponding cultures of leukocytes from only two unrelated subjects.

An increase in the ratio of female recipient to male graft donor metaphases in the cultures at the time of enhanced lymphocyte transformation indicated that proliferation of the graft recipient lymphocytes was responsible for the above findings. Unmixed, unstimulated control cultures grown in autologous, the other subjects plasma, or heterologous calf serum failed to support significant lymphocyte transformation. The role of humoral factors and relationship of the *in vitro* cellular responses to the *in vivo* homograft reaction are discussed.

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