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Immunological Marker Analysis of Mitogen-Induced Proliferating Lymphocytes Using BrdU Incorporation or Screening of Metaphases

Staphylococcal Protein A is a Potent Mitogen for $CD4^+$ Lymphocytes

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The proliferative effects of the mitogens phytohaemagglutinin (PHA), concanavalin A (Con A), pokeweed mitogen (PWM), and staphylococcal protein A (SpA) were investigated using two different methods which enable immunological marker analysis of proliferating cells: either surface marker labelling followed by BrdU incorporation or screening of metaphases after surface marker labelling. Therefore peripheral blood mononuclear cells from six healthy volunteers were stimulated with these four mitogens. Both PHA and Con A gave rise to more $CD8^+$ than $CD4^+$ proliferating cells. PHA, but not Con A, induced B-cell proliferation as well. PWM mainly caused T-cell proliferation. SpA also appeared to be a potent T-cell mitogen in addition to its capacity to induce B-cell proliferation. However, in contrast to the other mitogens SpA predominantly stimulated $CD4^+$ cells.

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In vitro mitogenic activation is widely used to investigate the functional properties of human peripheral blood cells. The evaluation of mitogenic responsiveness is especially of interest in cases of immunodeficiency. Most often mitogens such as the plant lectins phytohaemagglutinin (PHA), pokeweed mitogen (PWM), and concanavalin A (Con A) are used. Staphylococcus protein A (SpA), a protein present in the cell wall of many *Staphylococcus aureus* strains, can also be used to study mitogenic responsiveness [18, 28].

Incorporation of [^{3}H]thymidine by the proliferating cells is often used to measure the responsiveness of mononuclear cells (MNC) to these mitogens. Using this technique, it is not easy to investigate which specific cell subset responds. Approaches that have been used to determine which cells proliferate include separation of subpopulations either before or after in vitro culture by cell sorting, panning, or lysis [19, 25, 28, 32]. Other approaches make use of double-marker analysis of single cells. In this way the simultaneous presence of both certain differentiation markers and cell activation markers, such as HLA-DR, the transferrin receptor, and the IL-2 receptor, can be demonstrated [6, 17, 30]. However, these activation markers are probably not restricted to proliferating cells [20].

Definite markers for cell proliferation are those markers which are only expressed during specific phases of the cell cycle, e.g. the Ki-67 antigen and PCNA [8, 9, 21]. DNA content can also be

regarded as a marker for cell cycle phase [5, 22]. Furthermore, cells in S phase of the cell cycle can incorporate the thymidine analogue 5′-bromo-2′-deoxyuridine (BrdU), which can be detected by anti-BrdU monoclonal antibodies (MoAb) [4, 11, 24]. Finally, metaphase cells can be regarded as the best marker for the M phase of cell cycle.

We here describe two methods to determine the immunological phenotype of proliferating cells: BrdU incorporation and metaphases as markers for proliferation. We investigated which cells proliferate after stimulation with PHA, PWM, Con A, and SpA.

MATERIALS AND METHODS

Isolation of mononuclear cells. Peripheral blood (PB) MNC were obtained from heparinized venous blood of six healthy volunteers, aged 25–35 years, by Ficoll–Paque (1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density centrifugation. Cells were washed twice in RPMI 1640 culture medium without Hepes, supplemented with L-glutamine (4 mM) penicillin (100 IU/ml), streptomycin (50 μg/ml), and 15% heat-inactivated fetal calf serum (FCS). The FCS was specifically selected for its growth-supporting properties and low endogenous mitogenic activity.

Immunological marker analysis. To detect surface membrane markers, the cells were incubated with optimally titrated relevant MoAb as described [33]. The immunological markers used were CD20 (B1; Coulter Clone, Hialeah, Fla USA), CD37 (Y29/55; Dr H.K. Forster, Basel, Switzerland) to label B cells, CD3 (Leu-4; Becton Dickinson, San Jose, Calif., USA), CD4 (Leu-3; Becton Dickinson), and CD8 (Leu-2; Becton Dickinson) to label T cells; CD14 (My4; Coulter Clone) and CD15 (VIM-D5; Dr W. Knapp, Vienna, Austria) to label monocytic cells and myeloid cells respectively. Natural killer (NK) cells were labelled by use of CD16 (Leu-11b; Becton Dickinson) and CD57 (Leu-7; Becton Dickinson); in addition, CD25 (2A3, anti-IL-2-receptor; Becton Dickinson); CD71 (66IG10, anti-transferrin receptor; Dr M. van Rijn, Amsterdam, The Netherlands), and anti-HLA-DR (L243; Becton Dickinson) were used to label cells. The reactivity of the MoAb was visualized by use of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) antiserum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). The fluorescence stainings were evaluated with Zeiss microscopes equipped with phase-contrast facilities (Carl Zeiss, Oberkochen, FRG) [33].

Culture systems. For all cell culture experiments MNC were adjusted to a final concentration of 5×10^5 cells per ml. Cells were cultured in the same RPMI 1640 medium as was used for the isolation of MNC. Mitogens added in optimal concentrations were: PHA (100 μg/ml; Wellcome Diagnostics, Dartford, UK), Con A (100 μg/ml; Sigma Chemical Co., St Louis, Mo., USA), PWM (10 μg/ml; Gibco Laboratories, Grand Island, NY, USA), SpA (25 μg/ml; Pharmacia). As a control, cells were cultured without mitogens. For the measurement of [^{3}H]thymidine incorporation MNC were cultured in 96-well flat-bottom tissue culture plates (10^5 cells per well; Costar, Cambridge, Mass., USA). For the other experiments cells were cultured in tubes (3×10^6 cells per tube; Corning, New York, USA). Incubation was performed at 37 °C, at 100% relative humidity, and at a P_{CO_2} of 5%.

[^{3}H]thymidine incorporation. MNC from four donors were cultured for 2, 3, 4, 5, 6, and 7 days. [^{3}H]thymidine (specific activity 6.7 Ci/mmol; Amersham International, Amersham, UK) pulsing was for 6 h using 0.5 μCi per well. After the 6-h pulse the cells were harvested using an automatic cell harvester (Skatron, Lier, Norway). [^{3}H]thymidine incorporation was measured in a liquid scintillation counter (Packard, Downers Grove, Ill., USA). Each determination was performed in triplicate. The results are given as medians of the results obtained in the four donors.

Immunological marker analysis of metaphase cells. MNC from two donors were cultured with PHA or SpA for 2, 3, 4, 5, and 6 days. Cells were centrifuged and resuspended at a concentration of 10^7 cells/ml. Fifty microlitres of the cell suspensions was incubated at room temperature with 50 μl of the relevant MoAb. After 15 min the cells were washed twice and 50 μl of a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service) was added; to arrest the cells in metaphase 0.25 μg Colcemid (Gibco Laboratories) was added. After another 15 min the cells were washed in a hypotonic KCl solution (0.075M) and cytocentrifuge preparations were made (Nordic Immunological Laboratories, Tilburg, The Netherlands). The slides were put in atebrine (Gurr, Poole, UK) dissolved in ethanol 70% (vol/vol) for 10 min to fix the cells and to stain DNA. The atebrine staining was evaluated by using the same filter combination as used for FITC, and the expression of the immunological markers was evaluated by using a TRITC filter combination [33]. When possible, up to 100 metaphase cells were evaluated for each immunological marker. The percentage of cells in metaphase was determined by screening 2000 MNC.

BrdU incorporation and immunological marker analysis of BrdU$^+$ cells. MNC from the same four donors as were used for the [^{3}H]thymidine incorporation experiment were cultured with PHA, Con A, PWM, or SpA for 2, 3, 4, 5, 6, and 7 days. To label the cells in the S phase of the cell cycle, BrdU (Sigma) was added to a final concentration of 10 μM, 30 min before harvesting. After harvesting, cells were washed twice, incubated with MoAb for 30 min to label a surface membrane marker, and washed again. Subsequently 50 μl of a goat-anti-mouse Ig antiserum conjugated with colloidal gold particles of 5 nm size was added (Janssen Pharmaceutica, Beerse, Belgium). After 30 min the cells were washed twice and a cytocentrifuge preparation was made. The slides were air-dried, fixed in ethanol 70% (vol/vol) for 30 min, and denatured in NaOH 0.07 M for 2 min. After washing in $Na_2H_2BO_4$, the slides were incubated with a FITC-conjugated anti-BrdU MoAb (Becton Dickinson) for 30 min in a moist chamber. After two washings the immunogold-labelled slides were subjected to a silver enhancement procedure as

specified by Janssen Pharmaceutica. We used a silver development time of 25 min. For the visualization of the silver granules we used dia-illumination or, in the case of weak staining, epi-illumination with polarization filters. The percentage of cells having incorporated BrdU was determined by screening 400 MNC. For double-marker analyses with differentiation markers 200 $BrdU^+$ cells were evaluated.

After 6 days of culture cells were tested for cytoplasmic Ig (Cylg) expression as previously described [33]. Cytocentrifuge preparations were simultaneously incubated with an FITC-conjugated burro anti-human Ig-lambda antiserum and a TRITC-conjugated goat anti-human Ig-kappa antiserum (Kallestad Laboratories, Austin, Tex., USA).

RESULTS

Immunological marker analysis

The MNC fraction from the PB of all donors contained 5–10% B cells ($CD20^+$, $CD37^+$), 50–60% T cells ($CD3^+$) with a CD4/CD8 ratio which varied from 1.1 to 1.8, $<1\%$ granulocytic cells ($CD15^+$), and 15–25% monocytic cells ($CD14^+$). In addition, about 10% of the cells were positive for NK markers such as CD16 (Leu-11b) and CD57 (Leu-7). The percentages of positivity for HLA-DR reflected the sum of B cells and monocytic cells. Cells expressing CD71 (transferrin receptor) and/or CD25 (IL-2 receptor) were only detected at levels of $<1\%$.

[^{3}H]thymidine incorporation

The results of [^{3}H]thymidine incorporation on mitogenic stimulation are summarized in Fig. 1, left. Each point represents the median count from values obtained in four donors. The kinetics found in the four donors were similar to the kinetics shown in the compilation. The maximum PHA-induced [^{3}H]thymidine incorporation was found on days 3–5 (71,000–84,000 cpm). For Con A, maximum incorporation was found on day 4 in all donors (56,000–71,000 cpm). After PWM stimulation the maximum incorporation (23,000–52,000 cpm) was found on day 4 in one donor and on days 6 and 7 in the other three donors. For SpA the maximum incorporation was found on days 5 and 6 (63,000–113,000 cpm). Without the addition of mitogens, [^{3}H]thymidine incorporation was <1000 cpm up to day 5 and <3000 cpm on days 6 and 7.

Immunological marker analysis of metaphase cells

The results of the immunological marker analysis of metaphases after stimulation with PHA and SpA are summarized in Fig. 2. Each point represents the mean value of the counts obtained in two donors. The kinetics were similar in both donors. The maximum of PHA stimula-

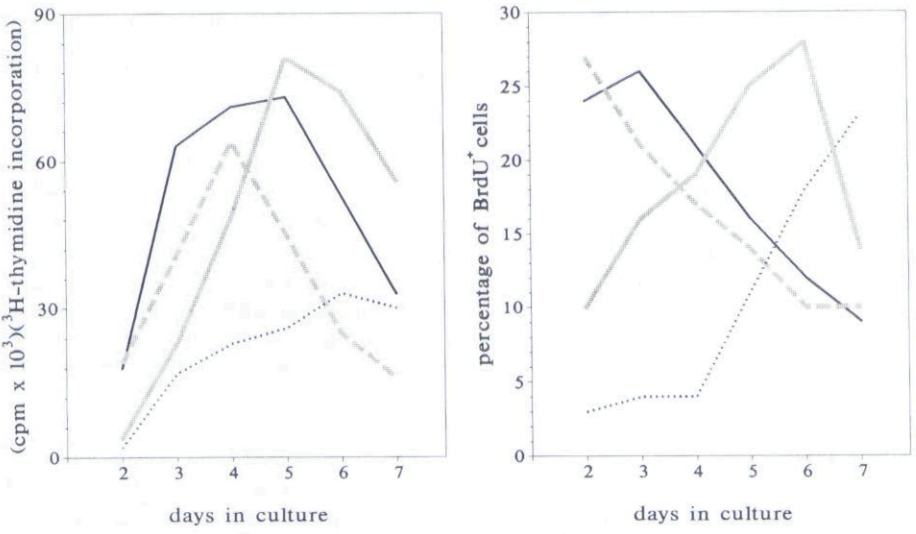

FIG. 1. [^{3}H]thymidine incorporation (left) and BrdU incorporation (right) of PBMNC upon mitogenic stimulation with PHA (——), Con A (▒ ▒), PWM (· · · · · · ·), and SpA (▒▒▒). Each point represents the median count from values obtained in four donors.

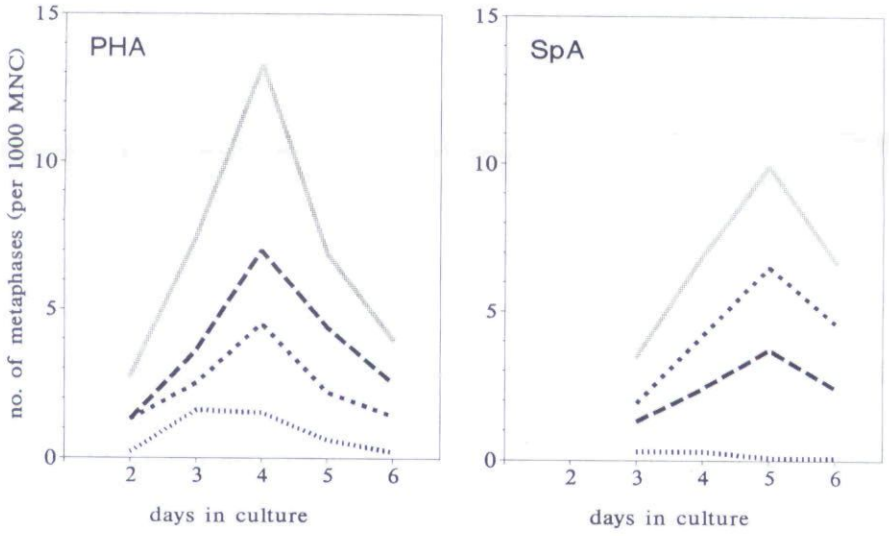

FIG. 2. Immunological marker analysis of metaphases after stimulation of PBMNC with PHA (left) and SpA (right). The total number of metaphases (▒▒▒) is shown as well as the number of $CD4^+$ metaphases (▪▪▪▪), $CD8^+$ metaphases (— — —) and $CD20^+$ metaphases (·····). Each point represents the average of two donors.

tion was found on day 4. On this day about 35% of the metaphases were $CD4^+$, about 55% were $CD8^+$, and about 10% were $CD20^+$. Upon stimulation with SpA the majority of the proliferating cells was $CD4^+$. The maximum percentage of cells in metaphase was found on day 5. At this point about 65% of the metaphases were $CD4^+$ and about 35% were $CD8^+$. A small proportion of proliferating B cells was detected on days 3 and 4.

BrdU incorporation and immunological marker analysis of $BrdU^+$ cells

The results of the incorporation of BrdU and the immunological marker analysis of $BrdU^+$ cells after stimulation with PHA, Con A, PWM, and SpA are summarized in Fig. 1, right, and Fig. 3 respectively. Each point represents the median of the results obtained in four donors. The kinetics found in the four donors were quite similar. Maximum stimulation, i.e. the highest percentage of cells in S phase of the cell cycle, was found for Con A on day 2, for PHA on day 3, for PWM on day 7, and for SpA on day 6 (Fig. 1, right). First we tried to label the surface membrane differentiation markers with a TRITC-conjugated antibody, however, probably because of the denaturation and fixation procedure necessary for visualization of BrdU, the TRITC signal was too weak. Therefore immunogold labelling with silver enhancement was used for labelling the surface membrane markers.

In all but one donor PHA and Con A gave rise to more $CD8^+$ than $CD4^+$ proliferating cells. In addition, distinct proliferation of B cells was observed on days 3 and 4 of PHA culture, while B-cell proliferation after Con A stimulation was minimal (Fig. 3, upper part). Stimulation with PWM resulted mainly in proliferation of T cells, which were slightly more often $CD4^+$ than $CD8^+$, and to a lesser extent in proliferation of B cells (Fig. 3, lower left). Upon stimulation with SpA in all donors the majority of the proliferating cells was $CD4^+$. B-cell proliferation was observed on days 2 and 3 only (Fig. 3, lower right).

Determination of other markers revealed that on all days of culture NK markers such as CD16 and CD57 were expressed on about 10% of the proliferating cells after PHA or Con A stimulation and about 5% of those cells after stimulation with PWM or SpA.

Activation markers were expressed by the $BrdU^+$ cells as well as $BrdU^-$ cells. These markers were not expressed by all $BrdU^+$ cells. Positivity for HLA-DR on the $BrdU^+$ cells was found in roughly the same percentages as positivity for CD20, indicating that these $HLA\text{-}DR^+$,

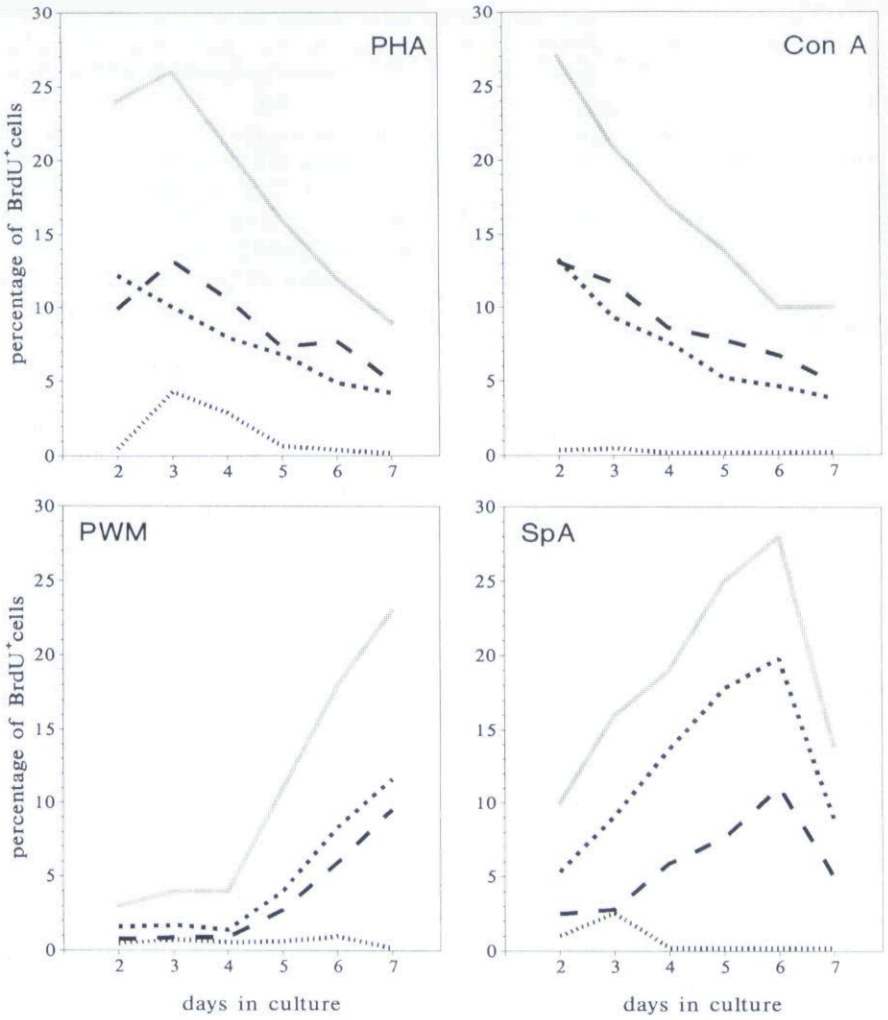

FIG. 3. Immunological marker analysis of $BrdU^+$ cells after stimulation of PBMNC with PHA (upper left), Con A (upper right), PWM (lower left), and SpA (lower right). The percentage of total $BrdU^+$ cells (▒▒▒) is shown as well as the percentages of $CD4^+$, $BrdU^+$ cells (▪▪▪▪), $CD8^+$, $BrdU^+$ cells (— — —), and $CD20^+$, $BrdU^+$ cells (·····). Each point represents the median count from values obtained in four donors.

$BrdU^+$ cells were probably proliferating B cells. CD71 (transferrin receptor) and CD25 (IL-2 receptor) were found on 60–80% of the $BrdU^+$ cells on day 3 and 30–60% of the $BrdU^+$ cells on day 7.

Without the addition of mitogens $<1\%$ of $BrdU^+$ cells were seen on days 2–6. On day 7 of the cultures, 2% of $BrdU^+$ cells were found in two donors, while in the other two donors about 7% of the cells had incorporated BrdU. Labelling of the cells revealed that on day 3 about 35% of the $BrdU^+$ cells were B cells and about 50% were T cells. In addition some monocytic cells ($CD14^+$) and NK cells ($CD16^+$, $CD57^+$) were proliferating. On day 7 the majority of the proliferating cells was $CD4^+$ T cells.

To investigate whether cells coexpressed CD4 and CD8, as has been reported by others [2, 30], double-marker analysis for these two markers was performed in two donors. It was not possible to perform a triple immunological marker analysis for BrdU and these two T-cell markers. In all

cultures $CD4^+$, $CD8^+$ cells were seen, however mostly the percentages were 1% or lower. In one donor, about 2% of $CD4^+$ $CD8^+$ cells were seen 6 days after stimulation with PHA.

After 6 days of stimulation with PWM about 1–5% of the MNC expressed Cylg. In the cultures stimulated with PHA, ConA, or SpA, and in the cultures without a mitogen, the proportion of $Cylg^+$ cells was less than 0.1%.

DISCUSSION

We here describe two different methods to determine the immunological phenotype of proliferating cells. The method which enables immunological marker analysis of metaphase cells was originally developed to combine cytogenetic analysis and immunological marker analysis at the single-cell level [13]. Using this method we found that PHA is a better mitogen for $CD8^+$ PBMNC than for $CD4^+$ PBMNC, while SpA mainly stimulates $CD4^+$ cells. To confirm this finding and to compare the kinetics of PHA and SpA with those of Con A and PWM, we used a technique which was less laborious and which enables the analysis of cells in S phase of the cell cycle, i.e. a double-marker analysis for BrdU incorporation and a differentiation marker.

Using the BrdU method, maximum percentages of cells in S phase were found on day 2 for Con A and on day 3 for PHA (Fig 1, right). On the other hand, using [^{3}H]thymidine incorporation, the kinetics for these two mitogens seemed to be different. The maximum incorporation for Con A and PHA was found on day 4 and day 5 respectively (Fig 1, left). This discrepancy is readily explained, since [^{3}H]thymidine incorporation is measured over the whole population of cells, while in the BrdU technique the percentage of cells in S phase is determined. Owing to the rise in the total cell number during culture, the absolute number of proliferating cells is maximal on day 4 or day 5 in Con A and PHA cultures, while the maximum proportion of proliferating cells is already found by day 2 or day 3 respectively. For SpA and PWM this discrepancy was not observed, probably because of the fact that the proliferation peak occurs at a later stage (day 5–6), when the culture medium becomes exhausted.

The results of the immunological marker analysis of $BrdU^+$ cells after PHA and SpA stimulation confirmed the results obtained by the metaphase technique. The preponderance of $CD8^+$ proliferating cells over $CD4^+$ proliferating cells after PHA stimulation was also found by others using different methods [3, 6, 30]. Con A also appeared to stimulate more $CD8^+$ cells than $CD4^+$ cells. This is in contrast with the findings of Persson & Johansson [25]. However, they separated the T-cell subsets prior to mitogenic stimulation. A major difference between PHA and Con A was the finding of a significant B-cell proliferation upon PHA stimulation, which was minimal after Con A stimulation. B-cell proliferation by PHA has previously been reported [4, 22, 23]. Interestingly this PHA-induced B-cell proliferation took place early (days 3 and 4), which suggests a direct effect of PHA on B cells and which supports the application of PHA to the culture of malignant B cells [26]. On the other hand, this early stimulation of B cells might be induced by early release of cytokines.

PWM is often used as a mitogen to test T-cell proliferation as well as T cell-dependent B-cell proliferation, but it turned out to stimulate primarily T cells. This confirms previous reported data [30]. In contrast to PHA and Con A, slightly more $CD4^+$ cells than $CD8^+$ cells were proliferating in the PWM cultures.

SpA appeared to be a potent T-cell mitogen, accompanied by some B-cell proliferation on day 3. Jacob & Rose [18] suggested using SpA as a mitogen for studying human T-cell function, however they did not test which T-cell subsets were proliferating. The consistent preponderance of $CD4^+$ over $CD8^+$ proliferating cells is in contrast to the results obtained with the other mitogens. Based on discontinuous bovine serum albumin density-gradient fractionation experiments, Sakane & Green [28] found that SpA-responsive T cells in human PB were different from the cells that respond to PHA. However, they did not perform immunological marker analysis.

Since SpA is such a potent mitogen for $CD4^+$ cells, it is interesting to use SpA for in vitro functional studies aimed at testing the proliferative capacity of this T-cell subset. It is well known that HIV type 1 infects and destroys $CD4^+$ cells [7, 12]. Decreased in vitro lymphocyte proliferative responses are well known in HIV-1-infected individuals and AIDS patients [29, 31]. The poor response of MNC from AIDS and pre-AIDS patients is not only ascribed to a decreased

number of $CD4^+$ cells, but the responsiveness of these cells also seems to be deficient [10, 16]. Publications on the use of SpA in functional studies of the MNC from HIV-infected persons are still lacking. Recently, Hofmann *et al.* [15] described the application of the mitogen-stimulated lymphocyte transformation response as a predictive marker for the development of AIDS and AIDS-related symptoms in persons infected with HIV. They found that, by comparing PHA, Con A, and PWM as mitogens, the risk of a worsened clinical condition was 55 times higher in seropositive men with a decreased PWM response than in other seropositive men. Later on, reduction of the proliferative responses to PHA and Con A took place. This sequential decrease in responsiveness to PWM, Con A, and PHA was also observed by Radvany *et al.* [27], but not by Alcocer-Varela *et al.* [1]. Hofmann *et al.* [14] explained this phenomenon by an at least partly preserved function of the PHA/CD2-dependent pathway. On the other hand, our data revealed that, in contrast to PHA and Con A, PWM stimulates more $CD4^+$ cells than $CD8^+$ cells. However, SpA turned out to be an even better mitogen for $CD4^+$ cells. Therefore, it may be rewarding to include SpA in functional studies of HIV-infected persons.

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