INTERLEUKIN 7 AS INTERLEUKIN 9 DRIVES PHYTOHEMAGGLUTININ-ACTIVATED T CELLS THROUGH SEVERAL CELL CYCLES; NO SYNERGISM BETWEEN INTERLEUKIN 7, INTERLEUKIN 9 AND INTERLEUKIN 4

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The effects of the interleukins IL-7 and IL-9 on cell cycle progression were investigated by conventional [^{3}H]thymidine incorporation and by the bivariate BrdU/Hoechst technique. Both IL-7 and IL-9 drive phytohemagglutinin-activated T cells through more than one cell cycle, but IL-7 was more potent on cell cycle progression than IL-9. Neither synergistic nor inhibitory effects were seen between various combinations of the lymphokines IL-7, IL-9 and IL-4 compared to each lymphokine alone. When T cells are activated with phytohemagglutinin for 3 days, all or most IL-4 responsive cells respond to IL-7 as well, whereas only a part of IL-7 responders are IL-4 responders. In contrast, when T cells are activated with phytohemagglutinin for 7 days, the quantitative data of the cell cycle distribution suggest that the population of IL-7 responders is at least an overlapping, if not a real subset of the population of the IL-4 responders.

T lymphocytes are regulated at multiple levels by a complex network of haematopoietic cells and cytokines. It has become clear that interleukin 2 (IL-2) is not the only factor controlling T cell growth, but several cytokines including interleukin 4 (IL-4), tumour necrosis factor, granulocyte/macrophage colony-stimulating factor, interleukin 1 (IL-1) and interleukin 6 (IL-6) are also involved.[1–5] In recent reports it has been shown that IL-7, initially described as a haematopoietic growth factor for early B lineage cells,[6] can act on T cell growth as well.[7] Interleukin 9 (IL-9), also known as T cell growth factor P40 in the mouse system, was originally described as a mouse T cell growth factor for certain T cell lines.[8,9] However, it was seen that IL-9 also mediates activities on erythroid progenitors and fetal thymocytes.[10,11]

In most reports data about T cell proliferation are obtained only by measurements of the [^{3}H]thymidine incorporation,[12–14] but little is known about cell kinetics of T cells stimulated with different lymphokines. We have shown that IL-2 as well as IL-4 can drive PHA-activated T cells through more than one cell cycle. Neither synergistic nor inhibitory effect on T cell proliferation was seen for the stimulation with both IL-2 and IL-4 as compared with the effect of IL-2 alone. The IL-4 responsive cells seemed to be a subset of the IL-2 responsive cells.[15] In view of the recently demonstrated potency of IL-7 and IL-9 on T cell growth, we investigated the cell kinetics of activated T cells stimulated with IL-7 and IL-9 and a putative synergism of these lymphokines with IL-4. Apart from the [^{3}H]thymidine proliferation assay we applied the bivariate BrdU/Hoechst technique for the examination of T cell proliferation. This method is based on incorporation of 5-bromo-2′-deoxyurdine (BrdU) into the DNA of proliferating cells. The quenching of Hoechst 33258 dye (Hoechst) fluorescence allows to separate cells according to the number of cell cycles (CC) the cells traversed during the observed time period. The unique attribute of this method is that it provides a clear distinction between different CC compartments of at least three consecutive CCs.

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RESULTS

The lymphokine-induced proliferation of the PHA-activated T cells was measured by means of [^{3}H]thymidine incorporation (Figs 1 and 2). Without the addition of any lymphokine, PHA-activated T cells showed only a negligible [^{3}H]thymidine incorporation, whereas with IL-9 a weak, but consistent proliferative effect on T cells activated with PHA for 3 days was seen. In contrast, both IL-4 and IL-7 had a strong proliferative effect on T cells activated with PHA for either 3 or 7 days. T cells preactivated for 3 days showed a higher [^{3}H]thymidine uptake when stimulated with IL-7 than with IL-4. The addition of IL-4, respectively IL-9, to IL-7 had neither significant synergistic nor inhibitory effects. In contrast, IL-4 was the most potent factor for the proliferation of T cells preactivated for 7 days with PHA and the addition of IL-7, respectively IL-9, to IL-4 did not influence the proliferating effect compared to IL-4 alone.

Preliminary data have shown that the detection of both [^{3}H]thymidine and the non-radioactive thymidine analogue BrdU incorporated in proliferating cells gave comparable results (data not shown). To clarify further the role of the different cytokines and their various combinations in their capacity to drive cells through different compartments of cell cycles, the proliferative response of activated T cells could be examined in more detail using a method labelling the cells continuously with bromodeoxyuridine in combination with Hoechst/ethidium bromide flow cytometry.

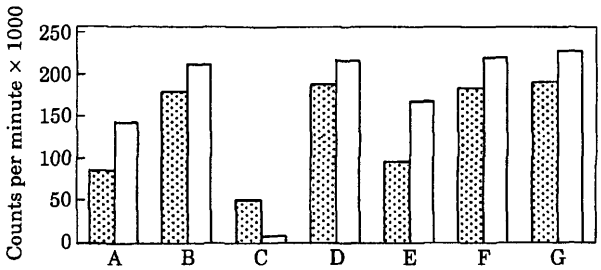

Figure 1. [^{3}H]thymidine incorporation in T cells PHA activated for 3 days.

T cells are activated for 3 days with PHA and then stimulated with (A) IL-4, (B) IL-7, (C) IL-9, (D) IL-4 and IL-7, (E) IL-4 and IL-9, (F) IL-7 and IL-9, (G) IL-4, IL-7 and IL-9 for 24 (■) or 72 h (□).

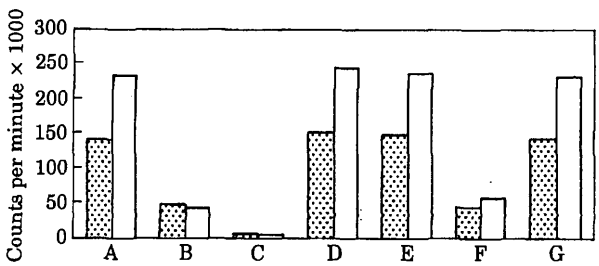

Figure 2. [^{3}H]thymidine incorporation in T cells PHA activated for 7 days.

T cells are activated for 7 days with PHA and then stimulated with (A) IL-4, (B) IL-7, (C) IL-9, (D) IL-4 and IL-7, (E) IL-4 and IL-9, (F) IL-7 and IL-9, (G) IL-4, IL-7 and IL-9 for 24 (■) or 72 h (□).

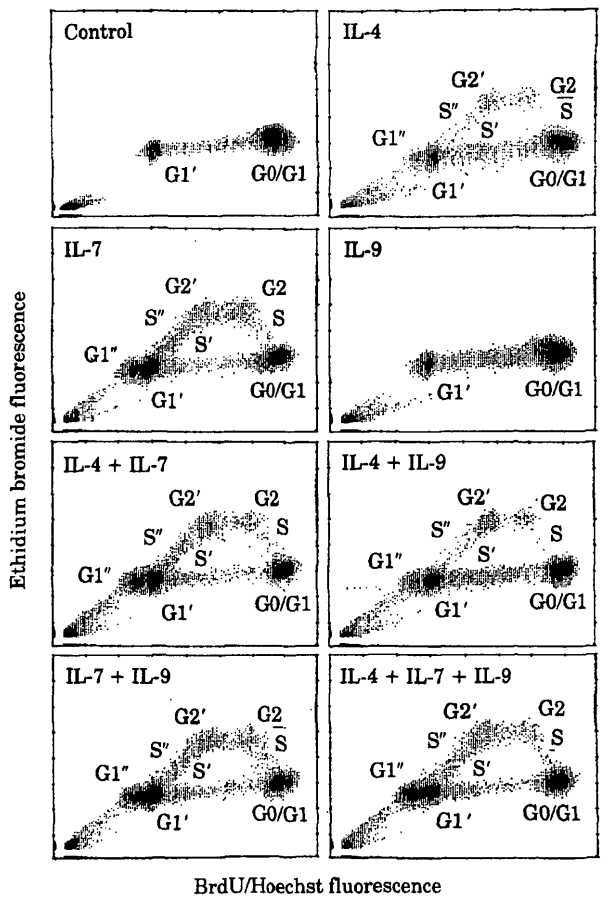

Figure 3. Bivariate Hoechst/ethidium bromide cytogram of PHA activated cell cultures continuously labelled with 100 μM BrdU in the presence of the indicated interleukins.

The abscissa displays BrdU-quenched Hoechst fluorescence while the ordinate shows unquenched ethidium bromide fluorescence. Nomenclature of the three CC displayed is G0/G1, S, G2; G1′, S′, G2′; G1″, S″, where the prime denotes the number of rounds of replication in the presence of BrdU.

Figure 3 shows representative cytograms of cultures preactivated for 3 days and then exposed to the different lymphokines for 72 h. The abscissa represents Hoechst fluorescence, which is quenched if BrdU is incorporated during S phase traverse of cells, whereas the ordinate shows BrdU-insensitive ethidium bromide fluorescence. Each dot represents an individual cell; clusters arise where cells with similar fluorescence concur. In the untreated control, nearly all cells remained in the resting state (G0/G1). In contrast, the cytogram of the IL-4 treated culture exhibits many cells left from the G0/G1 cluster (G1′). These cells have undergone one round of BrdU incorporation during S phase and halved their DNA content after traverse of the G2 compartment of the cell cycle. The trail moving to the right side and up from the G1′ cluster represents cells passing through a second S phase (S′) and a second G2 phase (G2′) in the second cell cycle. Due to the bilifary BrdU substitution, this trail no longer moves to the left, but still some additional quenching of Hoechst fluorescence is afforded during the second cycle S phase. Cells undergone a second mitosis appear to the left of the G1′ cluster (G1″). A few signals representing cells in the S phase of the third cycle can also be detected (S″). The panel representing the IL-7 treated culture elicits an even stronger proliferative response, whereas the IL-9 treated cells show little, but significant proliferation. Combinations of the interleukins including IL-7 lead to cytograms similar to that of IL-7 alone, whereas the combination of IL-4 and IL-9 leads to a similar cytogram to that of IL-4 alone.

Quantification of the data are shown in Tables 1 and 2. The data are corrected for the number of cell divisions a given cell has undergone and for the background. Most T cells activated for 3 days and then stimulated with the different lymphokines for 24 h remained in the G0/G1 phase of the first CC. When activated for 3 days and then stimulated for 72 h with the lymphokines, more than 35% of the cells stimulated with IL-7 alone or with a combination of lymphokines including IL-7 have left the first CC. In contrast, about 20% of the cells stimulated with IL-4 alone are found in the second or third CC. When stimulated for 24 h with IL-9 alone, about 1% of the 3 day PHA-blasts responded with a progression through cell cycles. The combination of IL-9 with IL-4, respectively IL-7, does not significantly influence the distribution of cells stimulated with IL-4, respectively IL-7, alone.

When activated with PHA for 7 days and then stimulated for 24 h with IL-4 alone or with a combination of lymphokines including IL-4, 20% of the cells have left G0/G1 of the first CC. IL-7 alone or the combination of IL-7 and IL-9 showed a weaker stimulatory effect than those achieved by IL-4 alone and combinations including IL-4. When stimulated for 72 h with IL-4 alone and combinations including IL-4, only about 45% of the cells remained in G0/G1 of the first CC. In contrast, about 65% of the T cells stimulated for 72 h with IL-7 alone, or with IL-7 and IL-9 were found in G0/G1 of the first CC. Stimulated with IL-9 alone, only a small percentage of the 7 day PHA-blasts were driven into the second, but not into the third CC.

TABLE 1. Cell cycle distribution of T cells, activated with PHA for 3 days and then stimulated with the indicated interleukins for 24, 48 or 72 h.

	First CC			Second CC			Third CC		
	G0/G1	S	G2	G0/G1	S	G2	G0/G1	S	G2
24 h									
Control	100	0	0	0	0	0	0	0	0
4	94.2	3.6	0	2.2	0	0	0	0	0
7	88.9	7.5	0	3.6	0	0	0	0	0
9	98.9	1.1	0	0	0	0	0	0	0
4 + 7	84.2	9.2	1.3	5.3	0	0	0	0	0
4 + 9	95.2	4.8	0	0	0	0	0	0	0
7 + 9	88.6	7.9	0	3.5	0	0	0	0	0
4 + 7 + 9	88.8	6.5	0.5	4.2	0	0	0	0	0
48 h									
Control	100	0	0	0	0	0	0	0	0
4	77.5	3.2	0	4.4	7.2	1.2	6.5	0	0
7	60.8	4.7	2.0	9.3	11.3	2.6	9.3	0	0
9	96.6	0	0.9	0.8	0.4	1	0.3	0	0
4 + 7	62.5	3.4	1.1	3.9	12.9	3.7	12.5	0	0
4 + 9	76.3	3.2	0	6.4	7.4	1.4	5.3	0	0
7 + 9	64.4	5.4	1.9	8	9.9	2.8	7.6	0	0
4 + 7 + 9	64.3	3.9	1.5	3.2	13.2	2.9	11.0	0	0
72 h									
Control	100	0	0	0	0	0	0	0	0
4	78.4	2.4	1.1	6.4	3.2	1.7	6.8	0	0
7	56.1	4.1	3	12.3	7.3	3.8	13.4	0	0
9	96.4	0.4	0	2.8	0	0	0.4	0	0
4 + 7	59.6	3.4	1.7	6.1	6.7	4.8	17.7	0	0
4 + 9	72.5	1.6	1.1	7.1	4.2	2.2	11.3	0	0
7 + 9	58.4	4.3	1.7	10	5.5	4.9	13.7	1.5	0
4 + 7 + 9	57.2	3.2	1.2	10.5	5.7	4.7	14.7	2.8	0

DISCUSSION

The stimulatory effects of different lymphokines on activation and proliferation of T cells can be examined with unprecedented precision by the bivariate BrdU/Hoechst technique. By this technique it is possible to obtain the distribution of stimulated cells in different CC compartments in at least three different CC.[16–18] In our test system, IL-4 as IL-7 and IL-9 drives activated T cells through more than one CC. So far, IL-9 was shown to stimulate proliferation of some murine Helper T cell clones. No direct response of human T cells to IL-9 was observed, moreover it

TABLE 2. Cell cycle distribution of T cells, activated with PHA for 7 days and then stimulated with the indicated interleukins for 24, 48 or 72 h.

	First CC			Second CC			Third CC		
	G0/G1	S	G2	G0/G1	S	G2	G0/G1	S	G2
24 h									
Control	100	0	0	0	0	0	0	0	0
4	82.5	3.4	9.1	5	0	0	0	0	0
7	95.4	0	1.3	3.3	0	0	0	0	0
9	100	0	0	0	0	0	0	0	0
4 + 7	83.7	0	11.5	4.8	0	0	0	0	0
4 + 9	75.2	9.1	9.7	6	0	0	0	0	0
7 + 9	95	0.5	3.4	1.1	0	0	0	0	0
4 + 7 + 9	75	10	9.4	5.6	0	0	0	0	0
48 h									
Control	100	0	0	0	0	0	0	0	0
4	53.4	2	0.9	11	15.4	2.9	14.4	0	0
7	77.8	2.3	1.8	11.9	4.3	0.4	1.5	0	0
9	91.5	3.4	2.9	1.1	1.0	0.2	0	0	0
4 + 7	51.7	1.6	0.6	8.6	16.2	2.6	18.7	0	0
4 + 9	55.9	1.6	0.1	8.3	13.7	2.7	17.7	0	0
7 + 9	75.3	3.5	1.2	15.2	4.3	0.5	0	0	0
4 + 7 + 9	58.9	5	0.8	12.7	14.5	1.5	6.6	0	0
72 h									
Control	100	0	0	0	0	0	0	0	0
4	44.8	0	2	7.5	9.9	1.2	34.6	0	0
7	62.5	1.6	2.6	24	3.6	0.6	5.1	0	0
9	88.6	0	1.1	8.7	1.2	0.4	0	0	0
4 + 7	45.9	0.1	2	8.5	6.6	2.4	34.5	0	0
4 + 9	43.8	0	2.3	10.8	10.2	0.9	32	0	0
7 + 9	62.7	0	3	20.1	4.4	0.2	9.6	0	0
4 + 7 + 9	48.1	0	3	9.3	7.1	1.6	30.9	0	0

seemed that human T cells need strong and prolonged activation for proliferation (Houssiau *et al.*, submitted for publication). We conclude that in our test system only a small proportion of cells was able to proliferate in response to IL-9. Interestingly, the proliferation was highest for T cells activated for 3 days and stimulated with IL-9 for 24 h. It remains obscure and will be addressed in future experiments whether the proliferating cells are T cells with special features or functions. Although IL-9 might be a T cell growth factor for some distinct T cell clones, it remains unclear what kind of preactivation the cells have had and what kind of additional signals will be needed for their proliferation. However, it seems to be clear that IL-9 will be no cofactor for the major known T cell growth factors including IL-2, IL-4 and IL-7 (data for IL-2 not shown).

Independent of the activation period (3 and 7 days), the percentage of the IL-7 responders stimulated to leave G0/G1 of the first CC remains constant with about 35% of the cells. These data are concordant with the findings of Welch showing that about 30% of IL-7 induced T cells entered into the S phase on day 3.[7] In contrast, the percentage of IL-4 responders increased from 30% to over 50% of the T cells activated for 3 and 7 days, respectively. Furthermore, neither synergistic nor inhibitory effects of IL-4 and IL-7 were seen on T cells activated for 3 or 7 days. Thus, one is tempted to conclude that in the system with 3 days activated T cells, the population of IL-4 responsive cells is at least an overlapping, if not a real subset of the population of the IL-7 responsive cells, whereas in the system with 7 days activated T cells, all or most IL-7 responders are also IL-4 responders, but only some IL-4 responders are IL-7 responders. Compatible to our data, Okazaki and Masuda[19,20] did not find synergistic or inhibitory effects of these lymphokines, whereas Bertagnolli[21] described an additive effect of IL-4 and IL-7 on murine thymocytes preactivated with ConA for 48 h. These differences may be due to the different steps of differentiation. Concordant to our results, Armitage[14] reported a stronger proliferative effect of IL-7 than of IL-4 on human T cells. In contrast to IL-4, IL-7 was shown to act on mature T cells in the absence of a comitogen[13] and it was found that freshly isolated T cells already express high levels of IL-7r while they express low levels of IL-4r.[22] Activation with PHA induces the expression of the IL-4r but has little or even negative effects on the level of the IL-7r expression.[22] These observations suggest that putative different activation states of T cells activated for 3 and 7 days, respectively, may be responsible for the discordant findings of proliferative responses to IL-4 and IL-7.

The bivariate BrdU/Hoechst technique is an important tool to uncover the interaction between different interleukins. Further investigations have to characterize the responsive cells for each factor to fully understand the mechanisms regulating T cell proliferation.

MATERIALS AND METHODS

Interleukins

The recombinant IL-4 protein was produced in *Escherichia coli* by the expression of the IL-4 cDNA.[24] T cells, activated with PHA for 7 days and then incubated with IL-4 for 72 h, were used to determine the biological activity of IL-4 using [^{3}H]thymidine incorporation (modified according to Yokota *et al.*)[1] Purified rIL-4 had a specific activity of 1.2 $\times$ 10^6 U/mg.

Recombinant human IL-7 was produced in *Escherichia coli* by the expression of the IL-7 cDNA.[24] The recombinant material is equivalent to that of the natural IL-7 except for the presence of an N-terminal methionine. The protein has a molecular weight of approximately 17.5 kDa. Peripheral blood lymphocytes, activated with PHA for 72 h, were used to determine the biologic activity (1 $\times$ 10^7 U/mg) in a [^{3}H]thymidine incorporation assay.

Recombinant human IL-9 (specific activity 6.6×10^6 U/mg in the MO7E cell test[10] was a kind gift of Dr Jaques van Snick (Ludwig Institute for Cancer Research, Bruxelles, Belgium).

Preparation of PHA Activated T Cells

Peripheral blood mononuclear cells (PBMC) from normal individuals were separated by Ficoll–Hypaque density gradient centrifugation. PBMC were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS, Gibco) (1×10^7 cells/ml) in the presence of 5 μg/ml phytohemagglutinin HA-15 (Wellcome, Research Triangle Park, NY) in a humid atmosphere wiht 5% CO_2 at 37°C for 3 and 7 days.

[^{3}H]thymidine Incorporation Assay

The PHA activated T cells were extensively washed, then 5×10^4 cells were placed in a 96-well flat bottom plate and cultured in 200 μl of RPMI-1640 containing 10% FCS in the presence of the different recombinant lymphokines. The culture was then pulsed with 0.5 μCi [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) for the last 6 h and then harvested onto glass fibre filters (Scatron, Oslo, Norway). The radioactivity was counted by liquid scintillation. All data are indicated as the mean counts per minute of at least triplicate cultures.

Bromodeoxyuridine Labelling

PHA-activated T cells were exposed to the interleukins as described above and concomitantly the culture medium was supplemented with 100 μM of bromodeoxyuridine (BrdU) and deoxycytidine. All culture plates were carefully wrapped in aluminium foil to avoid exposure to light of short wavelengths. After 24, 48 or 72 h of culture, cells were harvested under illumination with red light only. Until analysis, cells were stored in the dark at −20°C in culture medium supplement with 10% FCS and 10% dimethylsulphoxide.

Cell-staining and Flow Cytometry

After thawing, cell pellets were resuspended in a staining buffer containing 1.2 μg of Hoechst 33258 and 2.0 μg of ethidium bromide per ml of buffer.[17,18] Flow cytometric analysis was performed with an epi-illumination system of conventional design (ICP 22, Ortho Diagnostic Systems, Raritan, NJ) equipped with a mercury arc lamp (HBO 100, Osram, Berlin, FRG). Bivariate cytograms of Hoechst and ethidium bromide fluorescence were digitalized and recorded with a PDP 11/23 microcomputer (Digital Equipment Corporation, Maynard, MA). Cells belonging to three successive cell cycles can be distinguished due to BrdU quenching of Hoechst fluorescence.[16–18] By electronic selection of the signal clusters representing each cell cycle, rotation and deconvolution, conventional cell cycle distributions were obtained, which were fitted with a standard cell cycle algorithm (Phoenix Flow System, San Diego, CA). The cell numbers in each cell cycle were normalized to the percentage of original cells by correcting for the numbers of cell divisions by which cells originated.

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