DISCUSSION

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Infiltration of lymphocytes in spontaneously occurring tumors in humans and in various other animals have been observed in many occasions. Even poorly immunogenic tumors like MCA-102 and MCA-205 (MCA induced sarcomas in mice) are sometimes infiltrated by lymphocytes (Restifo et al., 1991; Marincola et al., 1994). Several authors suggested that the presence of Tumor Infiltrating Lymphocytes (TILs) within tumors might have strong prognostic significance (Mihm and Clark., 1971; Cochran, 1969; Hansen and McCarten, 1974; Underwood, 1974; Lauder and Aherne, 1972; Pardis et al., 1982; Sevennevig et al., 1985; McGovern et al., 1981; Poppema et al., 1983). TILs in most cases are $CD8^+$T cells and in presence of IL-2 differentiate into cytotoxic cells (Itoh et al., 1986). Belldegrun et al. (1989) noticed that TILs deprived of IL-2 *in vitro* stop producing mRNA for lymphokines and also lose their cytotoxic activity in long term culture. Several workers found that freshly isolated TILs from progressively growing tumors express weak cytotoxic activity against autologous or allogeneic tumors (Kurt et al., 1995; Keong et al., 1983; Miesher et al., 1986; Rubert et al., 1991). Being in close association with tumor cells *in vivo*, the immune status of TILs, whether sensitized, was tested in the present investigation.

TILs have been isolated from solid fibrosarcoma induced by an ascitic fibrosarcoma cell line maintained intraperitoneally. Isolation of TILs was achieved by centrifugation of cells through discontinuous percoll gradient and by velocity sedimentation at unit gravity through a constituted gradient. Of the two methods, percoll gradient method

produced better results. TIL enriched fractions collected from 55% percoll gradient yielded more TILs with less number of tumor cells as contaminant (Table 2). TILs obtained after velocity sedimentation was satisfactory (Fig. 8) but cell debris that descends along with TILs make the process of isolation complicated.

The number of TILs obtained from different tumors varied and there was not much correlation with the size of the tumors (Table 2). TILs may comprise of different cell types: T cells, NK cells, LAK cell precursors and other leukocytes like macrophages, eosinophils and neutrophils are also present in tumors (Bilik et al., 1989; Takagi et al., 1989; Ackermann et al., 1989). Out of all the cell types, T cells are considered to be most effective against malignant cells and thus the T cells infiltrating tumors can possibly be manipulated in ways that can trigger or amplify a patients insufficient immune reaction to combat malignancy. The major constituent in the TIL enriched fraction collected from 55% percoll gradient was T cells. The number of T cells varied from 45% to 80% with an average of 67% (Fig. 9). Such variation of T cells in TILs have been reported by Hayry and Totterman, (1978), Jondal and Klein, (1975), Sevennevig et al., (1978), Klein et al., (1981).

Different immunological responses of TILs were studied in reference to virgin and memory cells. T cells being the major constituent in the TIL population, Con A was used for activating the cells *in vitro*. In comparison to virgin lymphocytes, TILs rapidly transformed into blast cells after Con A treatment. The heightened blastogenic response was comparable to the blastogenic response of activated memory lymphocytes (Fig. 10 to 13). Blastogenesis of lymphocytes is the total

outcome of biochemical activities in preparation for cell division and functional differentiation. The kinetics of blastogenesis sharply differ in virgin and memory cells. Memory lymphocytes are known to mount rapid and vigorous immune response on restimulation and this is facilitated by enhanced expression of cell surface molecules like LAF-2, CD-2, involved in adhesion and signal transduction (Sanders et al., 1988; Sanders et al., 1989). Rapid transformation of TILs into blast cells probably suggests that TILs are in memory state. Almost 90% of the T cells in the TIL population transformed into blast cells.

On the other hand the peak of DNA synthesis by TILs was at 72 hrs (Table. 3) in comparison to the peak of incorporation of ^{3}H-TdR by virgin cells at 48 hrs (Table. 5). The memory cells generated with primary stimulus of Con A, incorporated no significant level of ^{3}H-TdR except at 24 hrs (Table 4). Tilman et al. (1990) suggested that the inability to proliferate is a feature of the memory cells and does not depend on the nature of stimulus used. While analyzing the proliferative response of total T cells and $CD4^{+}$ memory cells Morimoto et al. (1985) and Tedder et al. (1985) noted that memory cells exhibit lower proliferative responses following PHA stimulation. Tilman and co-workers (1990) observed that $CD8^{+}$ memory cells also exhibit minimal proliferation inspite of expressing rapid kinetics of biochemical activities. Reduced proliferative response of TILs have also been observed by Miescher et al. (1986). These observations clearly support the contention that the heightened blastogenic response associated with lower proliferative index of TILs as observed in this study indicate that the TILs might be in the category of memory cells. In this laboratory it has been shown earlier that blastogenesis and DNA synthesis do not

always go parallel after activation of murine lymphocytes with different doses of Con A (Chakravarty and Chaudhuri, 1983). However, it is to be noted that memory T cells and also TILs proliferate profusely *in vitro* when triggered with anti CD-3 antibodies or IL-2 (Byrne et al., 1989; Byrne et al., 1988; Itoh et al., 1988; Belldegrun et al., 1988; Heo et al., 1987; Yron et al., 1980).

It has been shown that DNA synthesis play a critical role during generation of cytotoxic response in virgin cells. As such DNA synthesis inhibitors like Hydroxyurea (HU) suppresses the cytotoxic differentiation of activated virgin lymphocytes significantly in comparison to the cytotoxic expression of activated virgin cells without HU treatment (Table 9). Jaroslav and Ortiz, (1971), Heininger and co-workers (1976) observed that virgin cells require at least one round of cell division to differentiate into cytotoxic effector cells. However DNA synthesis does not seem to be a prerequisite for memory cells since they are able to express cytotoxicity even after treatment with HU (Table 10). Cantor and Jandinski (1974) made similar observations and found that cytotoxic activity of sensitized lymphocytes in cultures were independent of DNA synthesis.

The prerequisite of cell division in virgin lymphocytes for functional differentiation possibly suggests that reorganization of proteins associated with DNA in chromatin occurs during cell division and it has something to do with the expression of genes in virgin lymphocytes. Seale (1978) suggested that the replication not only involves duplication of genetic material but also participates in the determination of its genetic disposition for functional expression. Thus the differentiation of many cell types upon receipt of a stimulus is

preceded by cell division (Seale, 1978). Furthermore, the alterations in the chromatin of virgin cells in course of functional differentiation seems to be stable after primary activation since memory lymphocytes acquire the ability to express cytotoxic activity even after inhibition of DNA synthesis.

Interestingly, cytotoxic expression of TILs (Table 11) is independent of HU treatment indicating their grouping with memory cells rather than with virgin cells. In all probability the conformational changes in the chromatin required to become effector cells have taken place in TILs while in association with tumor cells *in vivo*. As a consequence, HU could not inhibit significantly the cytotoxic response in TILs activated with Con A.

As HU treatment inhibits the functional differentiation of virgin lymphocytes upon activation, BUdR that suppress the activity of new genes also does not allow the maturation of virgin cells into cytotoxic effector cells (Table 12). But BUdR is ineffective in suppressing the cytotoxic function of memory cells in which the effector genes have once been activated (Table 13). Nedrud et al. (1975) and Chakravarty and Clark (1977) also observed that 50 μg/ml BUdR blocks the expression of new genes and inhibits cytotoxic expression in virgin lymphocytes. Zipfel and co-workers, (1989) demonstrated that more than 60 new genes are induced during primary stimulation of PBL in humans. Antigens or mitogen induced genes include c-fos, c-myc, IL-2 (Reed et al., 1986; Greene and Leonard, 1986) and others like IL-3, IL-4, IL-5, IL-6, GM-CSF, IFN, TNF which are differentially expressed in various cells (Yang et al., 1986; Cuturi et al., 1987; Yokota et al., 1986; Gurvey et al., 1986). The expression of these genes alongwith a few others, yet

to be identified are possibly prerequisite in case of virgin lymphocytes for differentiation to cytotoxic state and proceed further down to the development of memory cells.

The ability of memory lymphocytes to express cytotoxicity even in presence of BUdR (Table 13) implies that the set of genes required to function during expression of cytotoxic activity are already in a ready state to act. BUdR is known to affect the expression of new genes and that is why it is not inhibitory to the expression of cytotoxic function of memory cells.

By the count that BUdR has no significant inhibitory effect on the cytotoxic activity of TILs, the TILs may be categorized with memory cells (Table 14). Genes necessary for expression of cytotoxicity are likely to be in an active conformational state in TILs.

Thus DNA synthesis and new gene expression is a prerequisite for virgin lymphocytes to develop into effector cells. Cell division is often necessary for expression of genes in eukaryotic system (Seale 1978). Some changes in chromatin in course of cell division during primary activation seems to be stable that facilitates quick transcriptional activities in memory cells. This is apparent from the quicker and stronger immune response of memory cells during secondary activation.

These findings indicate that memory cells are likely to harbour more active genes than virgin lymphocytes. This was put into test by studying the sensitivity of chromatin to DNase I digestion. The sensitivity of chromatin to DNase I is particularly influenced by the state of packing of DNA with protein moieties within the chromatin. Weintraub and Frederick (1974) showed that chromatin treated with

trypsin become more susceptible to DNase I digestion. Both active and inactive genes in the chromatin are associated with nucleosomes (Lacy and Axel, 1975; McKnight and Miller, 1976; Foe et al., 1976) but the active portion of the chromatin are more susceptible to DNase I digestion than the bulk chromatin (Berkowitz et al., 1975). The sensitivity of the genes expressed in tissue specific manner to DNase I digestion was also tested. Weintraub and Groudine (1976) showed that active globin gene is preferentially digested by DNase I in chicken erythrocytes but not in chicken oviduct. Conversely works of Garel and Axel (1976) indicated, active ovalbumin gene in the oviduct but not in erythrocytes was easily digested. Thus the subtle difference in organization between active and inactive genes was hinted. Further DNase I was found to attack the altered conformational sites in the active region of the chromatin, primarily at promoters, enhancers (Georgopoulos et al., 1988; Hagman et al., 1990), locus-activating regions (Grosveld et al., 1987) and matrix attachment sites (Levy-Wilson and Fortier 1989). The active genes remain sensitive to DNase I even when they are not actively involved in mRNA synthesis (Garel et al., 1977; Flint and Weintraub, 1977). Thus genes once active are more susceptible to DNase I even when they are not being transcribed (Flint and Weintraub, 1977; Groudine et al., 1978). Moreover, van Assendelft and co-workers (1989) demonstrated the requirement of DNase I hypersensitive sites at the 5' end of the human β-globulin minilocus for its expression and transcription activity in tissue specific manner. Therefore, alterations in association of DNA and protein can be considered as a feature for gene expression (Gross and Garrard, 1988), that renders them susceptible to DNase I digestion. Pierre and coworkers (1989) made an interesting observation of differential

susceptibility of T cell receptor (TCR) gene during assembly in developing T cells. Right after the joining, the Diversity (D) and Joining (J) gene encoding for TCR are more susceptible to DNase I while the variable (V) gene, that still remains at a far away point and yet to join the D-J gene is relatively DNase I resistant.

In the present investigation chromatin of memory lymphocytes were more sensitive to DNase I digestion than virgin cells (Fig. 15 and 16). It reflects the exposition of more DNase I sensitive sites in the chromatin of memory cells. Electrophoretic profile of the chromatin fragment obtained after DNase I digestion at 5 and 10 mins also supports the above view (Fig. 17). DNase I digestion of the chromatin from memory cells produced more smaller fragments than that from virgin cells. This is likely due to presence of more DNase I sensitive sites in the chromatin of the former. The smaller fragments travel down the gels further than the fragments obtained from virgin cells.

With DNase I digestion at 30 mins, the mean index for DNA release for virgin and memory lymphocytes were 55.68% and 61.7% respectively (Fig. 18) where as these indices were at 43.7 and 51.47 during kinetic study of DNase I on chromatin (Fig. 15). This is because during kinetic study, aliquotes from same source were being collected at regular intervals, that also removed DNase I molecules from the sample. This reduced the amount of DNase I molecule entering the nuclei for digestion at further down the time points and might be the plausible cause of this discrepancy.

Presence of more DNase I sensitive sites in the chromatin indicates greater number of transcriptional sites in memory cells as the case is with other transcriptionally active genetic sites (Weintraub and

Groudine, 1976; Garel and Axel, 1976; Georgopoulos et al., 1988; Hagman et al., 1990; Grosveld et al., 1987; Levy-Wilson and Fortier, 1989; van Assendelft et al., 1989). These sites have likely been produced by alterations in conformational association between DNA and nucleoproteins during the replication of virgin lymphocytes upon first encounter with antigen or mitogen. The increased sensitivity of memory cell chromatin to DNase I also indicates the presence of more number of active genes in addition to the house keeping genes. The latter comprises of 15 to 20% of the total genes to maintain day to day activity within a cell (Weisbord, 1982). Thus, it seems that without being transcriptionally active, quite a good number of genes in memory cells remain in a ready state to express, thereby allowing them to respond vigorously on subsequent challenges by the antigens.

In this count, TILs populating the tumor site are similar to the memory cells. Chromatin from TILs exhibit more sensitivity to DNase I digestion than the chromatin of virgin lymphocytes; percentage of DNA release at 30 mins was 63.5% (Fig. 18). Thus TILs are likely to harbour more number of active genes in a ready to act state than virgin lymphocytes.

Cytotoxic ability of TILs also indicate that they are sensitized cells and are at functional state. Freshly isolated TILs as such express cytotoxicity and the cytotoxic index increases further with Con A stimulation (Table 6). Here we observed a subtle difference between TILs and memory cells. Criteria as discussed earlier indicated that memory and TILs are similar on several counts, but TILs, even without activation, were reasonably cytotoxic. Whereas memory cells were quiescent although harbouring genes at active conformational state

(Table 8). Memory cells need stimulation to express their cytotoxic potential within 24 hrs. Virgin lymphocytes on the other hand do never express cytotoxic activity unless they are activated and require at least 48 hrs to become cytotoxic effector cells (Table 7).

Different experiments performed in this study points that TILs are sensitized cells, in all likelihood against the tumor associated antigens (TAA). The presence of TAA on the tumor cells has been shown. Antibodies raised in rabbits against the tumor cells after adsorption with normal cell types specifically react with tumor cell surface antigens and definite precipitation lines develop in immunodiffusion assay (Fig. 4). Similar precipitation line does not develop against the spleen cell antigens or serum from normal mice. Interestingly, the rabbit α-TAA antibody form a precipitation line with ascitic fluid and with the serum from tumor bearing mice (Fig. 4 and 5). This indicates the presence of circulating TAA in the serum of tumor bearing mice. But no appreciable precipitation line developed when the serum from tumor bearing mice was tested against the tumor cell antigens or ascitic fluid (Fig. 6 A to C). This confirms the absence of antibodies in the serum of tumor bearing mice against the tumor associated antigens/tumor cells. This observation put in focus the cell mediated immunity playing a major role in curbing malignancy.

Earlier studies in this laboratory has revealed that adoptive transfer of polyclonally activated syngeneic T lymphocytes are quite effective in restricting tumor growth (Chakravarty and Maitra, 1990). Con A activated lymphocytes restricted the tumor development in the anterior eye chamber of mouse (Chakravarty and Maitra, 1983; Maitra and Chakravarty, 1990). T cells in TIL population are oligoclonal in

respect to T cell receptor (TCR) gene rearrangements (Steven et al., 1993; Belledgrun et al., 1989; Seung et al., 1993; Ferradini et al., 1993, Kurt et al., 1995). Polyclonal stimulation has an inherent advantage in stimulating any specific clone (Bevan and Cohn, 1975) without showing the antigen; sometimes the source of antigen might not be ethically justified for use, as with TAAs in malignant cells. In the present study, TILs were polyclonally activated with Con A and injected at the site of palpable tumor to judge the potential of these cells in restricting tumor growth.

It is evident from the slope of the curve (Fig. 19) that the growth rate of tumor in treated mice was considerably slower, especially during the first few weeks after immunotherapy than in control animals. The survivality of the treated mice also improved to some extent, and most interestingly, 37.5% of the mice receiving adoptive transfer recovered completely (Fig. 20). In comparison to the earlier findings with polyclonally activated lymphocytes used for adoptive immunotherapy (Chakravarty and Maitra, 1990), a single dose of 10^6 TILs produced better therapeutic effect. This was possible probably due to the specificity of the TILs against the tumor cells. There are more scopes to increase the efficacy of TILs. In another study in this laboratory, we have observed 67% complete recovery after adoptive transfer of Con A activated T cells in mice after surgical removal of the tumor (Chakravarty and Jha, 1996). Similar study with TILs might prove to be more effective in curbing malignancy.

Now a question may arise, inspite of the TILs being specifically sensitized as well as functionally active, why do they fail to restrict tumor growth *in vivo*. Probable reasons are many. The micro

environment of TILs *in vivo* is totally different from *in vitro* situation. Ray and Saha (1982) showed that immunosuppressive factors have a profound effect on the cytotoxic activity of T cells. Chakravarty and Maitra (1990) showed that polyclonally activated murine T cells are quite effective in controlling fibrosarcoma *in situ*. In their experiment, depletion of suppressor T lymphocytes by Cyclophosphamide (Cy) enhanced the activity of adoptively transferred T cells. Treatment of TILs with Cy produced similar results in other laboratories (Rosenberg et al., 1986; Spiess et al., 1987). Fernandez-Cruz et al. (1980) showed that subsets of T cells which are responsible for tumor regression *in vivo* did not show cytotoxic activity *in vitro*. Yoshino et al. (1992) noticed that tumor reactive cells accumulate in lung cancer tissue but fail to respond due to tumor derived factors. Thus the TILs expressing cytotoxicity *in vitro* may not always be effective *in vivo*. One round polyclonal stimulation of TILs *in vitro* prior to adoptive transfer might be an effective immunotherapeutic measure for malignancy.

Conclusion

The qualitative difference between memory and virgin lymphocytes were studied in reference to blastogenesis, DNA synthesis and sensitivity to Hydroxyurea (HU) and Bromodeoxyuridine (BUdR). The memory cells were quicker to enter into blastogenesis and do not show same degree of DNA synthesis as with virgin cells. HU as inhibitor for DNA synthesis does not suppress the differentiation of memory cells into cytotoxic cells as it suppresses the cytotoxic activity of virgin cells.

BUdR, at 50 μg/ml dose regime, has been shown to suppress the expression of new genes but not the genes expressed already. This drug again profoundly affects cytotoxic activity in virgin cells but not in memory cells.

These results suggests a qualitative state of difference in the chromatin of memory cells from that of virgin cells. Furthermore, it has been experimentally resolved in this study that the chromatin from memory cells is more susceptible to DNase I digestion than that of virgin lymphocytes. This indicates that memory lymphocytes have more number of active genes. These observations led us to suggest that there is a conformational change in nucleoprotein packing at the sites sensitive to DNase I digestion. This might have much to do with the state of memory.

Tumor infiltrating lymphocytes (TILs) isolated from murine fibrosarcoma have been found to be qualitatively similar to memory lymphocytes in terms of blastogenesis, DNA synthesis and cytotoxic activity in presence of HU and BUdR. DNase I sensitivity index of the chromatin from TILs was also of similar degree. By these criteria, TILs appear to be sensitized cells. Most probably, TILs being in close association with the tumor cells *in vivo* have become sensitized against the tumor associated antigens. As such they were effective in cytotoxic killing of the tumor cells even without any stimulation *in vitro*. TILs were also found to be quite effective in restricting malignant growth in adoptive transfer experiments.