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Lymphocyte transformation induced by autologous cells

XI. THE EFFECT OF AGE ON THE AUTOLOGOUS MIXED LYMPHOCYTE REACTION

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Summary. The autologous mixed lymphocyte reaction (MLR) was lower in newborn infants and healthy subjects over 65 years of age than in adults between the ages of 20 and 32. In contrast, the allogeneic MLR, although impaired in newborn infants, was normal in elderly subjects. The degree of impairment of the autologous MLR in elderly subjects was correlated with the impairment in the response of lymphocytes from elderly subjects to phytohaemagglutinin (PHA) and *Staphylococcus aureus* protein A (SPA). The percentage of autorosetting T cells and of T cells with the OKT4 phenotype was increased in elderly subjects. These findings are paradoxical as autoreactive T cells in young adults have been reported to be drawn from these two T-cell subpopulations.

INTRODUCTION

Cell-mediated and humoral immunity change during the normal ageing of experimental animals and man (Makinodan & Kay, 1980). Evidence for an age-associated defect in lymphocyte function is the observation that lymphocytes from persons over 65 years of age incorporate less tritiated thymidine ([^{3}H]-Tdr) than do lymphocytes from young subjects when cultured with plant lectins (Pisciotta, Westring, Deprey & Walsh, 1967; Weksler & Hutteroth, 1974; Inkeles, Innes, Kuntz, Kadish & Weksler, 1977). More recently, the proliferative response of T cells stimulated by autologous non-T cells, the autologous mixed lymphocyte reaction (MLR), has also been found to be altered with age. Fernandez & MacSween (1980) reported that the autologous MLR of ten subjects over 65 years of age (their sex was not specified) was significantly less than that of young adults less than 30 years of age. In contrast, Fournier & Charreire (1981) found the autologous MLR to be greater in women over 50 years of age than in women less than 50 years of age. No difference was found in the autologous MLR in young men and men over 50 years of age.

We have studied the autologous MLR in humans across a wide range of age. T lymphocytes were obtained from newborn infants, young adults between the ages 20 and 32, and healthy elderly adults between the ages of 65 and 86. The autologous MLR was significantly lower in newborn and old subjects than in young adults. The defect in the proliferation of T cells from old humans in the autologous MLR was compared with the impaired proliferative response of their T cells cultured with plant lectins. Finally, the cellular basis of the proliferative defect observed in elderly humans has been investigated. T-cell subsets were

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identified by monoclonal antibodies or by the interaction of T cells with autologous erythrocytes (A-RFC). Peripheral blood from elderly subjects had a greater percentage of T lymphocytes with the OKT4 phenotype and a smaller percentage of T lymphocytes with the OKT5 or OKT8 phenotype than did blood from young adults. The percentage of A-RFC was increased in the blood of elderly subjects compared to young adults.

MATERIALS AND METHODS

Subjects

Blood was obtained from healthy laboratory personnel between the ages of 19 and 32 and from healthy elderly persons between the ages of 65 and 86. All elderly persons were ambulatory volunteers who were free of diseases and not taking drugs known to effect the reactivity of lymphocytes. Umbilical cord blood was obtained at the time of delivery of full term infants.

Preparation of peripheral blood lymphocytes

Blood from the experimental subjects was drawn into a plastic syringe containing 10 u. of heparin (Upjohn Company, Kalamazoo, Mich.) per ml of blood. The blood was diluted with an equal volume of calcium and magnesium-free Hanks's balanced salt solution (HBSS, Microbiological Associates, Bethesda, Md) 35–40 ml of the diluted blood were layered over 12 ml of a sterile mixture of 4 vol. of Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) and 1 vol. of sodium diatrizoate (Hypaque, Winthrop Laboratories, New York) with a specific gravity of 1·078–1·080. Tubes containing the diluted blood layered on Ficoll–Hypaque were centrifuged at 400 *g* for 40 min at 20°. The cells removed from the interface were washed three times with HBSS containing 10% heat-inactivated foetal bovine serum (FBS, Microbiological Associates) and were collected by centrifugation at 250 *g* for 10 min at 20°. If the mononuclear cells were to be cultured they were then resuspended in RPMI 1640 (Microbiological Associates) with 100 u. penicillin/ml, 100 μg streptomycin/ml, 2 mm L-glutamine (Microbiological Associates), and 20% human AB serum. If the mononuclear cells were to be fractionated into T- and B-cell enriched populations they were placed into medium containing 20% FBS.

Fractionation of T- and B-cell populations

Sheep erythrocytes (SRBC) obtained from Flow Laboratories Inc., Rockville, Md., were washed twice with HBSS. The washed SRBC were suspended in HBSS at a concentration of $1{\cdot}5 \times 10^8$ cells/ml. Equal volumes of human lymphocyte and SRBC suspensions were mixed and centrifuged at 50 *g* for 5 min at 20° and maintained at 4° for 16 hr as described by Steel, Evans & Smith (1974).

The cell pellets were then gently resuspended, and 35 ml of the SRBC and lymphocyte suspension were layered over 12 ml of Ficoll–Hypaque in 28 × 106 mm polycarbonate tubes (Arthur H. Thomas Co., Philadelphia, Pa.) The cells were centrifuged at 400 *g* for 40 min at 4°. Non-T lymphocytes were collected from the interface. Rosetted T lymphocytes in the pellet beneath the Ficoll–Hypaque were treated with 0·83% ammonium chloride 0·17 M Tris buffer, pH 7·2 to lyse the SRBC and were collected by centrifugation (150 *g* for 10 min at 20°). The non-T and T-lymphocyte preparations were washed three times with HBSS and resuspended in the final culture medium: RPMI 1640 with 100 u. penicillin/ml, 100 μg streptomycin/ml, 2mM glutamine, and 20% heat-inactivated human AB serum at a concentration of 10^6 mononuclear cells/ml.

Lymphocyte proliferation stimulated by mitogens

Unfractionated peripheral blood lymphocytes (PBL) were cultured in triplicate in sterile multiwell round bottom plates (Linbro Chemical Co., New Haven, Conn.) in a total volume of 0·2 ml and incubated in the presence or absence of 20 μg purified phytohaemagglutinin (PHA, Burroughs-Wellcome Co., Research Triangle Park, N.C.), 100 μg concanavalin (Con A, Pharmacia Fine Chemicals, Piscataway, N.J.) or 100 μg *Staphylococcus aureus* protein A (SPA, Pharmacia Fine Chemicals). These doses of the three mitogens have been found to stimulate maximal thymidine incorporation in lymphocytes from young adults. Cultures were incubated for 4 days in a 5% CO_2/95% air humidified environment. DNA synthesis during the last 24 hr of culture was assessed by the incorporation of thymidine. One microcurie of methyl [^{3}H] thymidine ([^{3}H]-Tdr, specific activity 2 Ci/mM, Amersham/Searle Corp., Arlington Heights, Ill.) in 1 μl was added to each well. At the end of culture, samples were collected on glass fibre filter paper using a Titertek cell Harvester (Flow, Laboratories. Rockville, Md).

The glass fibre discs were placed into 15 × 45 mm vials and 2·5 ml of scintillant was added. These minivials were counted in a Searle ambient temperature liquid scintillation counter. The average thymidine incorporation in counts per minute of the

replicate cultures is given. The counting efficiency for tritium under these conditions was 34%. The mean incorporation of [^{3}H]-Tdr by PBL cultured alone was less than 500 c.p.m.

T-cell proliferation stimulated by non-T cells

Unidirectional autologous and allogeneic mixed lymphocyte reactions were established in triplicate in round bottom microtitre plates with 10^5 T cells and an equal number of irradiated (3000 rad from a cesium source) non-T cells as stimulators in a volume of 0·2 ml per well. MLR cultures were incubated for 6 days and DNA synthesis measured as described above. The mean incorporation of [^{3}H]-Tdr by T cells cultured alone was less than 500 c.p.m.

Quantification of human T cells which form rosettes with autologous erythrocytes

Autologous rosette-forming cells (A-RFC) were enumerated using the method of Fournier & Charreire (1977). Briefly 10^6 mononuclear cells prepared by Ficoll–Hypaque centrifugation (see above) were incubated with 64×10^6 autologous erythrocytes in 150 μl RPMI 1640 in the absence of serum at 4° for 16 hr. The cells were gently resuspended and A-RFC counted. The number of A-RFC per 10^3 nucleated cells is given.

Quantification of T-cell subpopulations

Cytofluorographic analysis of monoclonal antibodies with cell populations was performed by indirect immunofluorescence as described by Reinherz, Kung, Goldstein & Schlossman (1979a). In brief, mononuclear cells obtained by Ficoll–Hypaque centrifugation (see above) were resuspended in RPMI 1640 with 15% FBS, penicillin and streptomycin at a final concentration of 10^6 cells/ml. Adherent cells were depleted by incubating cell suspensions in plastic petri plates for 60 min at 37°. The non-adherent lymphocytes were removed and resuspended at a concentration of 10^7 cells/ml. One-tenth millilitre of suitably diluted monoclonal antibody (kindly provided by Dr G. Goldstein, Ortho Pharmaceutical Corporation, Raritan, N.J.) was added to 0·1 ml of the non-adherent cell suspension, mixed, and incubated at 4° for 30 min. The cells were collected by centrifugation, washed twice with 2·0 ml RPMI 1640 containing 5% FBS. After the second wash the cell pellets were resuspended in 0·1 ml of a 1:20 dilution of fluorescein isothiocyanate conjugated goat anti-mouse IgG (Meloy Laboratories, Springfield, Va.) and incubated for 30 min at 4°. Samples were washed twice in RPMI 1640 containing 5% FBS and resuspended in this medium at a concentration of 10^6 cells/ml. The cells were analysed using a FC 201 cytofluorograph (Ortho Diagnostics Instruments, Westwood, Mass.) interfaced to a Data General minicomputer. These cells were then analysed on the cytofluorograph, and the intensity of fluorescence per cell was recorded on a pulse height analyser. Background staining was obtained by substituting ascites fluid from a BALB/c mice injected with a non-producing hybrid clone.

Statistical methods

Statistical comparisons between groups (young and elderly subjects) was done using Student's *t* test. Since the distributions of responses to PHA, Con A, SPA, and autologous and allogeneic non-T cells were highly skewed, the data were first transformed by taking logarithms (base 10). The decline in parameter values after age 65 was then assessed using linear regression, with the significance level referring to the slope of the regression line. Relationships between parameters were assessed using Pearson correlation coefficients for the log-transformed data.

RESULTS

Effect of age on mixed lymphocyte reactivity in man

The maturation of many immune responses is incomplete at birth and many immune responses decline after mid-life. We have measured the response of T lymphocytes from newborn infants, young and elderly adults in mixed lymphocyte cultures. T lymphocytes from newborn infants were isolated from cord blood. T lymphocytes from adults were isolated from venous blood. The response of neonatal and adult T lymphocytes in the autologous or allogeneic MLR was compared (Table 1). Neonatal T lymphocytes incorporated significantly ($P < 0{\cdot}01$) less [^{3}H]-Tdr than did adult T lymphocytes in both the autologous and allogeneic MLR. Non-T lymphocytes from newborn infants were as stimulatory as were non-T lymphocytes from adults in the allogeneic MLR (data not shown) suggesting that the defect, at least in the allogeneic MLR, is attributable to the responding T-lymphocyte population.

Different results were observed when the response of T lymphocytes from elderly persons was compared with that of T lymphocytes from young subjects in mixed lymphocyte cultures (Table 2). Although the response of T lymphocytes from elderly adults was

Table 1. Impaired response of neonatal T lymphocytes in the autologous and allogeneic MLR*

Age of T-cell donors	[^{3}H]-Tdr incorporation (c.p.m./culture $\times 10^{-3}$) Autologous MLR	Allogeneic MLR
Newborn (5)	9·1 ± 1·9	15·2 ± 2·2
20–32 years (4)	13·5 ± 2·4	35·3 ± 6·4

* T lymphocytes (10^5) were cultured for 6 days with an equal number of either autologous or allogeneic non-T cells. Proliferation in culture was assessed by the amount of [^{3}H]-Tdr incorporated during the final 24 hr of culture. The number in parentheses indicates the number of subjects studied. Data are presented as the mean ± standard error.

Table 3. Impaired response of lymphocytes from elderly humans cultured with mitogens*

Age of cell donors (years)	[^{3}H]-Tdr incorporated (c.p.m./culture $\times 10^{-3}$) PHA	SPA
22–32	83·4 ± 8·3 (25)	63·2 ± 11·2 (43)
65–88	39·4 ± 2·5 (73)	38·2 ± 3·8 (48)

* PBL (10^5) were cultured for 4 days with either PHA, Con A or SPA. [^{3}H]-Tdr incorporated during the last 24 hr of culture was measured. The number in parentheses indicates the number of subjects studied. Data are presented as the mean ± standard error.

impaired to an extent comparable with that seen with neonatal T lymphocytes in the autologous MLR, T lymphocytes from elderly adults responded normally in the allogeneic MLR. The difference in [^{3}H]-Tdr incorporation in the autologous MLR from young or elderly adults was highly significant ($P < 0.001$). There was no difference in the autologous or allogeneic MLR with regard to the sex of the T-cell donor.

Relationship between impaired proliferative response of T lymphocytes from elderly subjects cultured with autologous non-T cells or with mitogens

Lymphocytes from elderly humans are impaired in their response to plant lectins. As has been previously reported, lymphocytes from elderly persons incorporated significantly less [^{3}H]-Tdr when cultured with the T-cell mitogens PHA ($P < 0.001$) than did lymphocytes from young adults (Table 3). The proliferative response of T lymphocytes from elderly donors induced by SPA, which stimulates both T and B lymphocytes, was also significantly ($P < 0.04$) less than that of young adults. Men and women, regardless of age, responded equally to these mitogens.

Table 2. Impaired response of T lymphocytes from elderly humans in the autologous MLR*

Age of T-cell donors	[^{3}H]-Tdr incorporation (c.p.m./culture $\times 10^{-3}$) Autologous MLR	Allogeneic MLR
20–32 years		
Male	13·1 ± 2·0 (23)	43·3 ± 4·8 (22)
Female	13·8 ± 2·6 (20)	42·3 ± 5·5 (19)
65–86 years		
Male	7·9 ± 1·8 (25)	36·5 ± 4·3 (25)
Female	6·3 ± 1·1 (26)	40·7 ± 5·1 (25)

* Lymphocytes (10^5) were cultured for 6 days with an equal number of either autologous or allogeneic non-T cells. Proliferation in culture was assessed by the amount of [^{3}H]-Tdr incorporated during the final 24 hr of culture. The number in parentheses indicates the number of subjects studied. Data are presented as the mean ± standard error.

As a large number of subjects over 65 years of age were studied, it was possible to demonstrate a progressive decline in response to PHA after the age of 65 (Fig. 1). An inverse correlation ($r = 0.36$, $P < 0.001$) was found between [^{3}H]-Tdr incorporation and age in this group. As shown in Table 4, [^{3}H]-Tdr incorporation in the autologous MLR in this group was significantly correlated with [^{3}H]-Tdr incorporated in cultures containing PHA ($r = 0.34$, $P < 0.01$); SPA ($r = 0.26$, $P < 0.02$); and allogeneic non-T lymphocytes ($r = 0.40$, $P < 0.001$). Thus, the impairment of T-cell response in the autologous MLR was correlated with the impaired response to SPA and PHA. Although the response of elderly T cells to allogeneic non-T lymphocytes was not impaired while the response to autologous non-T lymphocytes was, a correlation between the level of the auto- and the allogeneic MLR was found. Thus, despite the fact that the allogeneic MLR was not impaired in the elderly, individuals whose lymphocytes were most responsive in the allogeneic MLR were most responsive in the autologous MLR and conversely individuals whose lymphocytes were least responsive in the allogeneic MLR were least responsive in the autologous MLR.

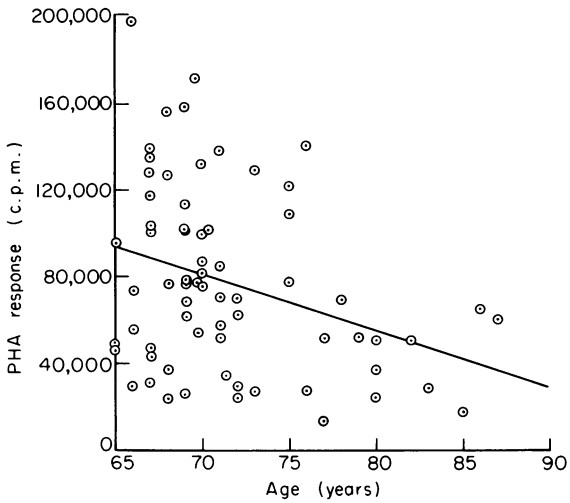

Figure 1. The correlation of mean [^{3}H]-Tdr incorporation by lymphocytes in triplicate cultures with PHA with age of lymphocyte donor is presented. Culture conditions are described in the Materials and Methods section. A linear regression derived from this data is shown.

The cellular basis of the impaired autologous MLR in elderly humans

The T cells which proliferate in the autologous MLR have been reported to be drawn from the population of T lymphocytes which form rosettes with autologous erythrocytes (Palacios, Llorente, Alarcon-Segovia, Ruiz-Arguelles, & Diaz-Jouanen, 1980) and which react with the OKT4 monoclonal antibody (Kozak, Moody, Staiano-Coico, & Weksler, 1981). Consequently, we have compared the percentage of T lymphocytes which react with autologous erythrocytes or with the OKT series of monoclonal antibodies in blood from young or old adults.

The percentage of lymphocytes which formed autologous rosettes was measured in twenty subjects over the age of 60 years and in twelve subjects less than 30 years of age (Table 5). The percentage of A-RFC in blood from old subjects was twice that from young subjects and this difference was highly significant ($P < 0{\cdot}002$). The percentage of A-RFC from men and women in both age groups was comparable.

Most investigators have found that the relative and total number of T lymphocytes do not change with age (Weksler & Hutteroth, 1974; Gupta & Good, 1979). Recently, it has become possible to identify subpopulations of human T lymphocytes using monoclonal antibodies (Reinherz *et al.*, 1979a; Reinherz, Kung, Goldstein & Schlossman 1979b, 1980a, 1980b). The percentage of lymphocytes reacting with the OKT3 antibody which identifies most T cells was the same in old and young humans. In contrast, the percentage of T lymphocytes reacting with the OKT4 antibody, which identifies a helper-inducer T-cell subset, was significantly ($P < 0{\cdot}002$) greater in elderly as compared with young adults (Table 6). Furthermore, the percentage of T lymphocytes reacting with the OKT5 or OKT8 antibody, which identify a suppressor-cytotoxic T-cell subset, was significantly ($P < 0{\cdot}002$) less in T cells from elderly as compared with young adults. Thus, while the autologous MLR is depressed in elderly humans, the percentage of autorosetting lymphocytes and T lymphocytes which react with the OKT4 monoclonal antibody is increased.

Table 4. Correlation of the responses to stimulation with mitogens and non-T cells*

Stimulus		SPA	Autologous non-T cells	Allogeneic non-T cells
PHA	*r*	0·336	0·338	0·408
	n	53	56	54
	P	0·014	0·011	0·002
SPA	*r*	—	0·255	0·179
	n	—	89	86
	P	—	0·016	0·099
Autologous non-T cells	*r*	—	—	0·401
	n	—	—	91
	P	—	—	0·001

* Correlations between the impaired responses of T lymphocytes to autologous non-T lymphocytes, SPA and PHA and the normal response to allogeneic non-T cells are presented. Statistical evaluation is described in the Materials and Methods section.

Table 5. Increase in autorosette-forming cells with age*

Age of subject (years)	ARFC/10^3 lymphocytes (%)	
	Male	Female
22–29	6·7 (7)	7·5 (5)
54–79	19·5 (4)	16·7 (16)

* Mononuclear lymphocytes were incubated with autologous erythrocytes in the absence of serum for 16 hr. Cells binding three or more erythrocytes were scored as A-RFC positive. The number in parentheses indicates the number of subjects studied.

Table 6. T-cell subsets in young and elderly humans*

Age of T-cell donors (years)	T cells identified (%)		
	OKT4	OKT5	OKT8
19–29 (18)	58·5	32·2	36·9
66–79 (18)	75·9	18·6	25·0

* PBL from healthy subjects were incubated at 37° with the indicated monoclonal antibody followed by a second incubation with fluorescein-conjugated goat anti-mouse IgG. Fluorescence emissions were then measured. The number of reactive cells is presented as a percentage of OKT3 reactive (i.e. total T) lymphocytes.

DISCUSSION

These studies demonstrate that T lymphocytes from newborn infants and elderly persons are less reactive in the autologous MLR than are T lymphocytes from young adults. T lymphocytes from neonates incorporated only one-half the [^{3}H]-Tdr incorporated by T lymphocytes from young adults in both the autologous and allogeneic MLR. Although T lymphocytes from subjects over 65 were similarly impaired in the autologous MLR, their T lymphocytes responded normally in the allogeneic MLR. No difference in the response of T cells from elderly male and female subjects was seen. Finally, we have found that aged subjects have a greater percentage of A-RFC and of lymphocytes which react with the OKT4 monoclonal antibody.

The impaired autologous MLR in subjects over 65 we observed confirms the observations of Fernandez & MacSween (1980). In contrast, Fournier & Charreire (1981) found no change in the autologous MLR in men over 50 years of age and an increase in the autologous MLR in women over the age of 50. The reason for the discrepancy is not obvious but may be attributable to differences in techniques utilized or in the populations studied. For example, Fournier & Charreire (1981) used PBL as the responding cell population while Fernandez & MacSween (1980) and we used T lymphocytes isolated by E rosettes. Neither Fernandez & MacSween (1980) nor we studied subjects between 50 and 65 years of age. It is possible that there is a transient increase in the autologous MLR of females between these ages. However, our data does not support the suggestion by Fournier & Charreire (1981) that the increased response of T lymphocytes from elderly women in the autologous MLR is due to an increased number of A-RFC. We find increased numbers of A-RFC in the lymphocytes of elderly subjects despite an impaired autologous MLR.

The increased number of A-RFC and of OKT4 positive lymphocytes in elderly subjects is paradoxical, as the T cells which proliferate in the autologous MLR have been reported to be drawn from the A-RFC (Palacios *et al.*, 1980) and OKT4 positive (Kozak *et al.*, 1981) population. Two possible explanations should be considered. The higher percentage of A-RFC in elderly subjects may reflect the increased number of cells of early T lineage which accumulate as thymic function declines with age. In the absence of adequate thymic activity, these cells may not acquire the capacity to respond in the autologous MLR.

It is also possible that these cells can respond in an autologous MLR but that this response is inhibited by increased non-specific suppressor activity which has been observed in elderly animals (Goidl, Innes & Weksler, 1976). It should be noted that recent studies have suggested that the OKT4 positive T cells are not homogeneous and contain both helper T cells and inducer T cells which activate suppressor activity in the OKT8 population (Thomas, Sosman, Irigoyen, Friedman, Kung, Goldstein & Chess, 1980). Thus, the increased numbers of OKT4 positive cells may result from an increase in the suppressor-inducer subpopulation of OKT4 positive cells.

The impairment in the autologous MLR in elderly

subjects is correlated with their impaired response to plant lectins. This suggests that the T lymphocytes which respond to autologous non-T cells may be drawn from the same T-cell population as are lymphocytes that respond to plant lectins. As alloreactivity is well maintained in elderly subjects, it is likely that autoreactive T cells belong to a population distinct from alloreactive T cells. A dissociation in auto- and alloreactivity has also been observed in patients with systemic lupus erythematosus (Kuntz, Innes & Weksler, 1979) and with infectious mononucleosis. (Moody, Casazza, Christenson & Weksler, 1979).

Finally, the significance of the impaired autologous MLR in neonatal and elderly should be considered. Much evidence suggests that the autologous MLR plays an important role in regulating immune reactivity. Thus, a number of immune effector functions including helper activity (Hausman & Stobo, 1979), suppressor activity (Innes, Kuntz, Kim & Weksler, 1979), and cytotoxic T-cell activity (Vande Stouwe, Kunkel, Halper & Weksler, 1977) are generated during the autologous MLR. Furthermore, the autologous MLR is impaired in several autoimmune diseases: Sjögren's Syndrome (Miyasaka, Sauvezie, Pierce, Daniels & Talal, 1980), systemic lupus erythematosus (Sakane, Steinberg & Green, 1978), and biliary cirrhosis (James, Elson, Waggoner, Jones & Strober, 1980).

The generation of suppressor activity during the autologous MLR may be one immunoregulatory mechanism that prevents the expression of autoimmunity. T-cell tolerance induced by low concentrations of self-determinants may be the primary mechanism that prevents autoimmunity (Allison, Denman, & Barnes, 1971). Suppressor cells may be an important 'backup' system to maintain self-tolerance. Thus, the impairment of the autologous MLR in elderly humans combined with the known decrease with age in the susceptibility to T-cell tolerance (DeKruyff, Rinnoy Kan, Weksler & Siskind, 1980) may explain the increased expression of autoantibodies (Hallgren, Buckley, Gilbertsen & Yunis, 1973) in the elderly. Infants who also have impaired autologous MLR may be protected from autoimmunity by their greater susceptibility to T-cell tolerance.

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