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Increase of cross(auto)-reactive antibodies after immunization in aged mice: a cellular and molecular study

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Summary. Aging in both humans and laboratory animals is often accompanied by an increase in autoreactive antibodies. Here we report that immunization with a bacterial antigen determined a marked increase of cross-reactive antibodies in aged but not in young mice. This phenomenon was observed in the aged individuals of two different mouse strains (Balb/c and C57BL/6) after a single injection of lyophilized vaccine from *Streptococcus pneumoniae* R36a (Pn) that express the immunodominant epitope phosphorylcholine (PC). These data were then confirmed by the analysis of cross-reactivity of anti-PC monoclonal antibody (mAb) generated from Pn-immunized young and aged Balb/c and C57BL/6 mice. Most of the anti-PC mAb from aged mice showed a broad spectrum of cross-reactivity with a panel of self and non-self antigens, while none of the mAb from young mice did so. We also showed that a genetic shift, in the V_H/V_L gene repertoire of anti-PC antibody, appeared to take place in aged mice and that aged mAb displayed a decrease in antibody affinity for the free hapten PC as compared to the young ones. We interpret these data as showing that immunization at advanced age may be linked to the production of cross-reactive antibodies and that this event may be related to the increased incidence of autoantibody in the aged.

Keywords: aging, autoimmunity, antibody, B cell, antibody affinity, immunoglobulin genes, vaccination

One of the most important effects of aging is the marked decline in immune responsiveness (Makinodan *et al.* 1971; 1977). However, in the aged immune system of humans and laboratory animals, the age-related decrease of immune response is accompanied by an increase in autoreactive antibodies directed against cellular constituents and products of various kinds of cells (Hirokawa 1985; Dighiero *et al.* 1982; Goidl *et al.* 1989). Autoantibodies, however, are not always related to clinical manifestations, for autoreactive antibodies have been found in the sera of healthy humans (Dighiero *et al.* 1982; Guilbert *et al.* 1982; Logtenberg *et al.* 1986) and autoreactive B cell clones have been isolated from young mice (Dighiero *et al.* 1983). Thus, it appears that autoreactive clones are normally present in the immune system and it has been hypothesized they may play a physiological role in immune regulation and defence (Dighiero *et al.* 1982; Cohen & Cooke 1986; Darwin *et al.* 1986). However, in the present study we show that immunization with a bacterial antigen determined a massive increase in cross-reactive antibodies in aged but not in young mice. The experiments were performed using young (3–4 month) and aged (18–22 month) mice of different genetic backgrounds (Balb/c and C57BL/6) and the increase in cross-reactive antibodies was seen in both strains tested. In our experiments mice were

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immunized with vaccine from *S. pneumoniae* R36a (Pn) and we evaluated the antibody response to the immunodominant epitope PC. The anti-PC response has been extensively studied, both at cellular and molecular level, in young/adult mice (Claflin *et al.* 1984; Perlmutter *et al.* 1984) and for this reason it represents a useful tool for investigating the age-related modifications of antigen-specific antibody response. The study was initially performed at cellular level by evaluating, in Pn-immunized mice, the response to PC and to other unrelated antigens. The presence of a high proportion of cross-reactive antibodies was visualized by ELISPOT assay at the level of the single antibody forming cell (AFC). The study at cellular level was then confirmed by the analysis of the properties of the anti-PC mAb generated from young and aged individuals. We observed that virtually all mAb from aged mice displayed a significant binding with different antigens. Furthermore, we demonstrated that the altered specificity of the aged mAb was accompanied by a molecular shift in the V_H/V_L gene families utilization. The difference between young and aged mAb gene repertoire was very distinctive owing to the paucity of clones of young repertoire, in which most if not all PC antibody was encoded by a single V gene family for each H and L chain, respectively V_H-S107 and V_κ-22. Furthermore, the mAb from aged mice showed a marked decrease in average antibody affinity for the free hapten PC. At present, the increase in cross-reactive antibody and the genetic shift in the aged mice can not be related to each other, but we hypothesize that the augmentation of antibody with altered specificity after immunization could be related to the increased incidence of autoantibody in the aged.

Materials and methods

Animals

Young (3–4 month) Balb/c and C57BL/6 mice were purchased from Charles River and aged in our facility. The animals were maintained in a clean, restricted-access animal room. All the mice were autopsied and only one aged mouse was found to be bearer of a tumour and so eliminated from the analysis. The in-vivo antibody response was evaluated using groups of 7–9 (immunized) or 4 (non-immunized) mice.

Antigen and immunization

Mice received a single i.p. injection of 0.25 μl of saline suspension containing 50 μg of lyophilized vaccine prepared according to a previously described protocol (Kelsoe *et al.* 1980). Briefly, pneumococci were grown from frozen stock in 10% DMSO and 10% FCS at −80°C. Frozen cells were plated onto trypticase soy agar with 5% sheep blood (Becton Dickinson Microbiological Systems, Cockeysville, MD) and the growing colonies were then inoculated in Todd–Hewitt broth (Difco Laboratories, Detroit, MI). Then the early log phase pneumococci were harvested by centrifugation (3000 r.p.m./20 min), washed twice with cold PBS, resuspended in PBS and then lyophilized overnight.

Enzyme-linked immunospot (ELISPOT) assay

Mice, immunized or not, were sacrificed on day 6 after the injection of the antigen and the spleens were removed aseptically. Spleen cell suspensions were prepared individually in PBS 2% FCS and the number of splenocytes per spleen was enumerated by Bürker's chamber. After washing, the cells were resuspended in ELISPOT medium: RPMI 1640; 5% FCS; Cytodex 1 (all reagents from Sigma, St Louis, MO).

A modified version of the ELISPOT assay (Nicoletti & Borghesi 1992) was used for the enumeration of antibody response at the level of the single AFC. Membrane filters were coated with various antigens at 50 μg/ml in PBS. Phosphorylcholine–bovine serum albumin (PC-BSA) was prepared according to Inman *et al.* (1973) using aminophosphocholine and BSA. Bovine haemoglobin (Hb), hen's egg lysozyme (HEL), mouse transferrin (mTrans) and mouse haemoglobin (mHb) were purchased from Sigma. The nitrocellulose filter discs (0.45 μm pore size, and 47 mm diameter, Scleider & Schuell, Keene, NH) were placed in sterile Petri dishes and incubated with antigens overnight at 4°C in a humid chamber. Membranes were then rinsed with cold PBS, blocked with PBS containing 2% BSA and 2% casein for 3 hours at 37°C, washed with cold PBS and then incubated with splenocytes at various numbers (ranging from 10^5 to 10^6 splenocytes/disc) for 4–6 hours at 37°C, 5% CO_2. After the incubation the filters were then treated with PBS/10 mM EDTA for 10 min at room temperature and then rinsed with PBS. Filters were then incubated overnight with horse-radish peroxidase labelled antibody against the mouse κ-chain (Southern Biotechniques Associated, Birmingham, AL). Filters were then washed with washing solution (PBS, 0.05% Tween 20, 0.02% SDS, and 1% casein). Finally membranes were incubated with HRP colour development reagents (BioRad, Richmond, CA) for 5–10 min and the reaction was stopped with deionized water. Blue spots were scored. The ELISPOT assay was performed in duplicate.

The previous enumeration of splenocytes from each individual cell suspension enabled us to calculate the antibody response per spleen.

Production of anti-PC hybridomas

PC-reactive mAb were generated from young and aged mice by fusion of splenocytes, 6 days after Pn immunization, with the SP 2/0-Ag14 cells (Shulman *et al.* 1978) by polyethylene glycol-mediated fusion in accordance with previously described technique (Galfre *et al.* 1977). The cells were seeded into microtitre plates (Costar, Cambridge, MA) at 10^4 cells/well with 10^5 thymic feeder cells/well. Supernatants from wells that were positive for cell growth were screened for mouse Ig and for reactivity with PC-BSA by standard ELISA technique. Cells from wells containing PC-binding Ig were then cloned by limiting dilution at 0.3 cells/well. After another screening for Ig secretion and for PC reactivity the clones were then subcloned under the aforementioned stringent conditions. The results obtained from all recovered mAb are presented. These were obtained from separate fusions from two young and two aged Balb/c and one young and one aged C57BL/6.

Screening and protein purification of anti-PC hybridomas

Culture supernatants were screened for PC-binding by standard ELISA. Briefly, 96-well microtitre plates were counted with 50 μl of PC-BSA (50 μg/ml) by overnight incubation at 4°C in a humid chamber. The remaining binding sites were saturated by incubation with PBS 2% BSA for 3 h at 37°C. The plates were then washed and 50 μl of culture supernatant was added to each well in duplicate. After an overnight incubation the plates were carefully washed with PBS and then incubated with 50 μl of β-galactosidase conjugated goat anti-mouse κ-chain antibody (Southern Biotechnology Associated, Birmingham, AL). The plates were incubated overnight and the β-galactosidase activity was then measured using *o*-nitrophenyl-β-D-galactopyranoside (Sigma) as the chromogen. Cells from wells positive for PC-binding were then cloned by limiting dilution (0.3 cells/well), retested and cloned once again.

Clones that were positive for PC binding were expanded in culture flasks and the supernatants were precipitated in 40% ammonium sulphate. The precipitates, resuspended in PBS, were then further purified by affinity chromatography on PC coupled to glycyltyrosyl-Sepharose followed by elution with 10^{-3} M PC chloride and extensive dialysis against PBS according to previously described procedures (Inman *et al.* 1973; Strickland *et al.* 1987).

Cross-reactivity of hybridomas

The purified Ig were screened by ELISA test, performed as described above, with a panel of different antigens: bovine haemoglobin (Hb), hen's egg lysozyme (HEL), cytochrome C (Cyto C) from horse heart, mouse transferrin (mTrans) and mouse haemoglobin (mHb). The background binding to BSA-coated plates was determined for each clone at concentrations from 10^1 to 10^4 ng/ml. The binding was considered significant when it exceeded the mean background plus × 10SD. Bindings that required more than 1 μg/ml of Ig were excluded.

Determination of antibody affinity

The affinity of purified mAb for PC chloride was determined by the ligand-induced changes in the immunoglobulin fluorescence according to Jolley and Glaudemans (1974) using a Perkin-Elmer LS-5B spectrofluorometer.

Molecular analysis of anti-PC hybridomas

Total RNA from mAb was purified as previously described (Chirgwin *et al.* 1979). Briefly, hybridomas were grown to mid-log phase and lysed by guanidine isothiocyanate buffer. Samples were then layered onto 5.7 M CsCl and centrifuged for 21 h at 174 000 ***g***. The RNA pellet was precipitated in ethanol, dried in a vacuum centrifuge and resuspended in diethylpolycarbonate-treated water. The concentration of RNA was determined by OD, and 8 μg of total RNA from each clone was fractionated on a 1.4% agarose–formaldehyde gel and transferred to MSI nylon filter (Fisher Scientific, Springfield, NJ). After transfer the filter containing the RNA was baked in a vacuum at 80°C for 2 h. Each filter contained a positive control and several negative control RNA. The size of the hybridizing band was determined by noting the position relative to that for the ribosomal RNA visualized in the gel. The filters were then prehybridized in 5 × SSPE (SSPE 0.15 M NaCl, 0.01 M NaH_2PO_4 and 0.0012 M EDTA), 2% Denhardt's, 0.1% SDS and 250 μg/ml denaturated herring sperm DNA for at least 3 h at 43°C. The filters were hybridized with 10^6 c.p.m./ml of nick-translated probe in the same solution with 10% dextran sulphate for 16 h and washed twice with 5 × SSPE and 0.1% SDS. A final wash of 0.1 × SSPE and 0.1% SDS was performed at the hybridization temperature for 15 min before exposure at −70°C with an intensifying screen.

Table 1. Antibody response (AFC/spleen) of young and aged Balb/c mice

Antigen	Age (months)	Non-immunized	P†	Pn-immunized	P†
Hb	3–4	145±10*		125±9	
	18–22	135±9	—	750±98	0.02
HEL	3–4	180±11		140±11	
	18–22	145±15	—	830±112	0.025
mHb	3–4	125±7		140±9	
	18–22	141±14	—	600±45	0.02
mTrans	3–4	135±8		150±11	
	18–22	155±10	—	815±120	0.015
PC-BSA	3–4	120±12		85000±14500	
	18–22	135±16	—	105000±38000	—

Non-immunized and Pn-immunized young and aged BALB/c mice were tested for antibody response to PC and to unrelated antigens by ELISPOT assay, 6 days after immunization.

* Mean from 8 mice/group ± SD.

† The *P*-values were calculated by Wilcoxon Rank test and were considered significant at $P \leqslant 0.05$. The *P*-values were omitted when not significant.

Table 2. Antibody response (AFC/spleen) of young and aged C57BL/6 mice

Antigen	Age (months)	Non-immunized	P†	Pn-immunized	P†
Hb	3–4	125±10*		130±9	
	18–22	125±9	—	600±68	0.025
HEL	3–4	160±15		140±16	
	18–22	165±25	—	530±100	0.03
mHb	3–4	130±13		125±12	
	18–22	121±10	—	715±65	0.01
mTrans	3–4	140±13		150±9	
	18–22	155±10	—	590±80	0.025
PC-BSA	3–4	130±12		38000±9700	
	18–22	120±6	—	14000±3800	0.018

Non-immunized and Pn-immunized young and aged C57BL/6 were tested for antibody response to PC and to unrelated antigens. (See legend Table 1 for details.)

Statistical analysis

Statistics were performed by the Wilcoxon Rank test. The *P* values were considered significant at $P \leqslant 0.05$.

Results

Primary anti-PC response of young and aged mice

Young and aged Balb/c and C57BL-6 mice were immunized i.p. with a single dose of Pn vaccine and the response was evaluated on day 6 after the challenge by ELISPOT assay. The response of Balb/c mice is summarized in Table 1. Young and aged non-immunized mice showed no difference in antigen-binding. An average number of plaques visualized using the normal (non-immune), young and aged, splenocytes ranged from 100 to 200 per spleen regardless of the protein used for the filter coating. This non-specific background probably represents the sensitivity limit of the ELISPOT assay. Young immunized mice showed a marked increase only in the anti-PC antibody response and we did not observe any increase in antibody binding to unrelated antigens. In contrast, the pattern of the antibody response appeared to be different in the aged Pn-immunized mice. Antibodies that bound to unrelated proteins were seen, regardless of the antigen used. The anti-PC response in aged mice was consistently higher than that observed in young Balb/c, even though we did not detect any significant difference.

Also, the anti-PC response in aged BL/6 mice is characterized by large amounts of antibodies that bound to unrelated protein antigens (Table 2). Interestingly, in this mouse strain, the magnitude of the anti-PC response in the aged displayed a dramatic decline as compared to the young ones (respectively 14000±3800 and 38000±9600 AFC, $P = 0.018$), showing that the magnitude of the anti-PC response in aging mice differs according to the mouse strain.

Thus, it appeared that immunization in aged mice is linked to the production of 'promiscuous' antibody.

Molecular analysis of anti-PC hybridomas from young and aged mice

Hybridomas producing monoclonal antibody directed against PC were generated, as described in Materials

Table 3. Molecular analysis of anti-PC hybridomas from young and aged donors

Hybridoma no.	Strain	V_H gene	V_κ gene
18–22 months			
13	Balb/c		
12	Balb/c		
15	Balb/c	S107	
13B	Balb/c	7183	
16	Balb/c		
14	Balb/c	S107	
22	C57BL/6	J558	
6	C57BL/6	J558	
7	C57BL/6	J558	
30	C57BL/6		
31	C57BL/6		
3–4 months			
39	Balb/c		22
40	Balb/c	S107	22
41	Balb/c	S107	22
42	Balb/c	S107	22
43	Balb/c	S107	22
44	Balb/c	S107	22
45	Balb/c	S107	22
46	Balb/c	S107	22
47	Balb/c	S107	22
9	C57BL/6*	S107	22
29	C57BL/6	S107	22
49	C57BL/6	S107	22
50	C57BL/6	S107	22

V_H and V_L gene utilization by PC-reactive mAb from young and aged mice. RNA from hybridomas was tested for its ability to hybridize to DNA probes representing three different V_H families (V_H S107; 7183; J558) and the V_κ 22 gene family. RNAs that failed to hybridize with the probes used (but did hybridize with the appropriate $C\mu$, $C\gamma$ or $C\kappa$ DNA) were assumed to express V genes of other families.

* Isotype γ; isotype μ in all other cases.

and methods, on day 6 after the primary immunization with Pn vaccine. In order to determine the V_H/V_L gene repertoire, a Northern analysis was performed using purified RNA from each anti-PC clone. We summarize the results in Table 3. Twelve mAb from young mice did use the V_H-S107 gene family and only one mAb (no. 39 from Balb/c) did not. In contrast, only two out of 12 mAb from aged donors used the V_H-S107, while the other clones used different gene families. The molecular screening was performed using DNA probes representing three different V gene families (V_H S107, 7183 and J558). However, the difference appeared to be even more striking in regard to the V_κ gene repertoire. All the mAb from young donors expressed the V_κ-22 gene family while none of the aged mAb did so. It appears that a genetic shift takes place in either the H or L chain repertoire in the aged mice of both strains tested.

Antigen binding and affinity of mAb

A study of antigen binding with a panel of self and non-self proteins was then carried out using the purified mAb. The ELISA test was performed using an antibody concentration of 1 μg/ml; bindings that required more than this amount of protein were not considered in our analysis. In Figure 1 is illustrated the antigen reactivity of a typical young (mAb 43, Figure 1a) and a typical aged (mAb 13, Figure 1b) anti-PC hybridoma. The aged PC-reactive hybridomas showed a broad spectrum of cross-reactivity

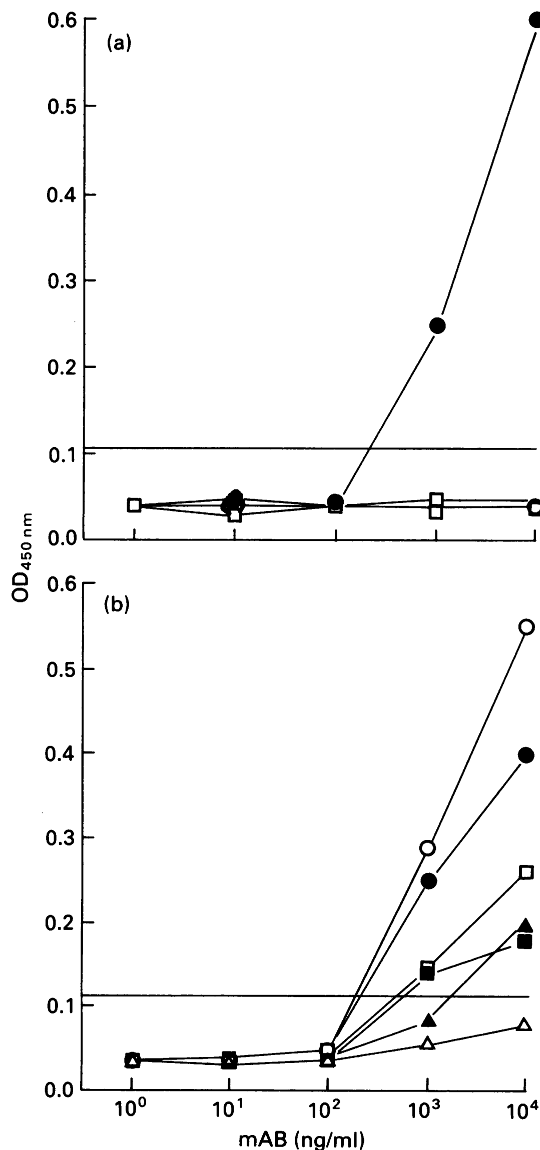

Figure 1. Example of antigen-reactivity of typical mAb(s) from a, young (mAb 43) and b, aged (mAb 13) mice. The hybridoma from young donors reacted only with ●, PC, while that from the aged mice displayed a remarkable binding, at 1 μg/ml, to different antigens (●, PC; ○, HEL; □, Hb; ▲, Cyto C). The horizontal line represents the background value calculated as described in the Materials and methods section.

Table 4. Summary of antigen cross-reactivities and antibody affinities for the free hapten PC of mAb from young and aged mice

	Antigens						
Hybridoma no.	PC	HEL	Hb	Cyto C	mTrans	mHb	PC Affinity ($K = 10^5$ M^{-1})
18–22 months							
13	+	+	+	+	−	−	0.45
12	+	+	+	−	−	−	0.18
15	+	−	−	−	−	−	0.20
13B	+	−	+	−	+	−	0.70
16	+	+	−	−	−	−	0.30
14	+	−	−	−	−	−	0.50
22	+	−	−	+	−	−	0.30
6	+	−	−	−	−	+	0.40
7	+	−	+	−	−	+	0.25
30	+	+	+	−	−	+	nt
31	+	+	+	−	+	−	nt
3–4 months							
39	+	−	−	−	−	−	1.5
40	+	−	−	−	−	−	nt
41	+	−	−	−	−	−	3.8
42	+	−	−	−	−	−	1.0
43	+	−	−	−	−	−	4.0
44	+	−	−	−	−	−	2.8
45	+	−	−	−	−	−	1.0
46	+	−	−	−	−	−	3.2
47	+	−	−	−	−	−	2.5
9	+	−	−	−	−	−	1.9
29	+	−	−	−	−	−	2.0
49	+	−	−	−	−	−	nt
50	+	−	−	−	−	−	2.4

The antigen-binding (+) of the affinity purified mAb was determined by ELISA test.
nt, Not tested.

against self and non-self antigens (Table 4). In contrast, none of the mAb generated from young mice bound to unrelated antigens. Thus, we confirmed that the antibody response, following antigen administration, in aged mice is characterized by a high incidence of cross-(auto)reactive antibody.

Next, we assessed the antibody affinity of PC-reactive mAb for the free hapten (Table 4). The affinities of the aged mAb for PC were about sixfold lower as compared to the young ones (average at $K = 10^5$ M^{-1}, 0.36 for aged mAb and 2.3 for young mAb).

Discussion

This study demonstrates that immunization of aged mice with Pn vaccine induced a significant increase of antibodies binding to self and non-self antigens. This phenomenon appeared to be linked to the immunization, for the normal (non-immunized) young and aged mice showed no difference in cross-reactivity at cellular level. These observations confirmed data obtained by other investigators (Kohtaro & Hirokawa 1988) reporting that there was no significant difference in the frequency of cross-(auto)reactive antibody-producing clones between non-immunized young and aged mice, although larger amounts of natural autoantibodies were present in the sera of old mice. These data suggested that in young animals the production of naturally occurring autoantibodies was silenced by some suppressive mechanism and the age-related impairment of this suppressive mechanism could be responsible for the expansion of cross-reactive clones at serum level.

Monoclonals from aged donors clearly showed a marked decline in average antibody affinity for the free hapten PC. This result is fully in accord with the notion that, at an advanced age, antibody displays a decline in affinity for the antigen (Kishimoto *et al.* 1976; Goidl *et al.* 1976; Weksler *et al.* 1978). This event may reflect alterations in the mechanism of antibody affinity maturation in the aged immune system.

A molecular shift in the antibody repertoire seems to take place in the aged mice. The anti-PC antibody was encoded, in both Balb/c and C57BL/6, by different V_H and V_L gene families as compared to young animals. All the anti-PC clones generated from young mice, with the exception of one clone, were encoded by a single gene family for H (V_H-S107) and L (V_κ-22) chains, while the gene repertoire of hybridomas from aged donors appeared to be extremely heterogeneous. The explanation of the genetic shift remains speculative. It could be that aged B

cells may not use the V_H-S107/V_K-22 gene combination because of intrinsic molecular defects. However, elegant experiments performed by Miceli *et al.* (1990) demonstrated that in randomly (LPS)-activated B cells the V_H and V_L gene families are expressed stoichiometrically in both young and aged mice. Thus it appeared that the genetic shift is probably influenced by changes in the mechanisms of selection and maturation of the aged B cells.

Remarkably, data obtained at the cellular level by ELISPOT assay were confirmed by the analysis of cross-reactivity performed by ELISA test using the purified anti-PC mAb. Increase of cross-reactivity in antigen-specific mAb(s) from aged mice was also observed by other investigators (Martin-McEvoy & Goidl 1988). They immunized young and aged C57BL/6 with trinitrophenyl(TNP)-Ficoll and observed that more than 70% of the aged mAb from old mice showed cross-reactivity to self antigens. It seems that the increase of cross-reactive antibodies after immunization in aged mice takes place regardless of the antigen used for priming. Furthermore, it has been reported (Goidl *et al.* 1990) that the anti-TNP antibody response of aged mice is also characterized by changes in V_H genes' repertoire. Thus, it is possible that changes in V genes' repertoire and altered antibody specificity in aging may be interrelated.

The age-related modifications that we have described here are likely to have a significant influence on the immune function for we have previously demonstrated (Nicoletti *et al.* 1993) that passive administration of anti-PC antibody obtained from aged donors did not protect young recipients against lethal injections of pneumococci, while antibody from young donors did so. The decreased protective capability of PC antibody and the immunization-related increase of cross-(auto) antibodies in the aged suggest that a passive administration of functional 'young' antibodies, rather than vaccination, may represent the best strategy in order to protect the elderly against infectious diseases.

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