Effect of Age on the Gastrointestinal-Associated Mucosal Immune Response of Humans

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Age-related changes in gastrointestinal-associated mucosal immune response have not been well studied. Thus, we investigated the effect of age on this response and compared these responses to those of peripheral immune cells. Saliva, blood, and intestinal biopsies were collected from young and old healthy subjects to determine immunoglobulin (Ig) levels and to isolate peripheral blood mononuclear cells, intraepithelial lymphocytes (IELs), and lamina propria lymphocytes (LPLs). Although subject age did not influence the level of total IgA found in saliva, IgA levels in serum increased ($p < .05$) with age. Older subjects’ peripheral blood mononuclear cell proliferation and IL-2 production were significantly lower than those of young subjects. LPLs from older subjects produced significantly less IL-2 in response to all stimuli than did that from the young. IEL’s ability to proliferate and produce IL-2 was not affected by subject age. Thus, LPL but not IEL demonstrated an age-related decline in immune function similar to that seen in peripheral lymphocytes.

The well documented age-associated decline in the systemic immune response (1) contributes to decreased resistance to pathogens, leading to increased morbidity and mortality as a result of infections among the elderly (2). The gut-associated immune system is the largest component of the human immune system. The gastrointestinal-associated mucosal immune response (GAIR) has adapted to its position at the interface between environment and the organism by developing features that are structurally and functionally distinct from other components of the immune system (3). For example, the GAIR is capable of mounting a humoral immune response, but the dominant immunoglobulin (Ig) isotype produced is IgA combined with a secretory component, whereas IgG is the major immunoglobulin found in the circulation (4). Because the GAIR is so distinct, many principles established for systemic immune response cannot be applied directly to those occurring in mucosal tissue (5). Because of the inherent difficulty in obtaining human tissue, the few existing published experiments either have used rodents or have dealt with secretory immunity. Little is known regarding the GAIR with respect to age-related changes. To our knowledge, the few studies that have investigated age-related changes in cellular immunity have been limited to rodents and the results are contradictory, reporting either decline or no change in cellular immunity with age (6–9).

For understanding the human GAIR, animal studies are of limited value because the human GAIR differs in many respects, such as lymphocyte composition, from that of other species. Diet is one of the many factors that accounts for these differences. To date, age-related changes in cellular GAIR have not been investigated. This should be addressed, because the elderly population are particularly susceptible to gastrointestinal infections, morbidity caused by foodborne pathogens, and inflammatory and neoplastic bowel diseases, which suggests that the mucosal immune defenses in the aged are compromised (10,11).

The GAIR is composed of both organized and “scattered” lymphoid populations. Intestinal mucosal lymphocytes are defined by their location within the epithelium. Lamina propria lymphocytes (LPLs), the scattered lymphoid population, reside just under the basement membrane in the intestinal villi; intraepithelial lymphocytes (IELs), also scattered, are located just above the basement membrane between the columnar epithelial cells. Interspersed among the villi are lymphoid nodules known as Peyer’s patches (PPs). Each of these three populations has distinct cellular components and functions and together form a complex, interconnected network. Representing the “organized” lymphoid tissue in the GAIR, PP are the most extensively studied lymphoid population. Age-related changes in cellular constituents of PPs from rodents have been reported, but the effect of aging on LPLs and IELs from humans is unknown. The objective of this study was thus to determine the effect of age on IEL and LPL immune response. In other human studies, the GAIR tissue from patients undergoing surgery for bowel resection or morbid obesity has been used. In this study, however, the GAIR was acquired from nonsurgical biopsies of normal intestinal tissue from healthy, nonobese humans (12,13). As a result of the small amount of tissue available for study, only those functional immune parameters that...
most consistently have exhibited age-related changes (i.e., lymphocyte proliferation and IL-2 production) in the systemic immune response were investigated.

**Materials and Methods**

**Subjects**

Young (20–40 years) and older (≥65 years) men and women were recruited. Candidates with gastrointestinal disorders, atrophic gastritis, a history of gastrectomy, high blood pressure, unusual dietary habits, and excessive alcohol intake, users of H2 blockers, and smokers were excluded. Candidates taking nutritional supplements, aspirin, anti-inflammatory medications, or antibiotics were likewise excluded, unless they had refrained from taking these substances for at least 1 month prior to the start of the study. Subjects underwent a preadmission screening, which consisted of a physical examination, psychosocial assessment, and routine blood clinical chemistries. As a way to check for bleeding tendency, platelet count and prothrombin were also determined. Those with a tendency to bleed as determined by these parameters were excluded. Seventeen young (15 men and two women) and seventeen older (16 men and one woman) individuals completed the study. Subjects checked into the Metabolic Research Unit of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University the night before the experiment. The following morning, following a 12-hour fast, the intestinal biopsy, blood, and saliva were collected.

**Blood Collection and PBMC Separation**

Blood was collected in foil-wrapped vacutainer tubes with sodium heparin and maintained at room temperature until processed. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-paque (Pharmacia Biotech, Piscataway, NJ) density-gradient centrifugation as previously described (14). PBMCs were cultured in endotoxin-free Rapid Prototyping and Manufacturing Institution (RPMI) 1640 (Sigma Chemical Co., St. Louis, MO) media supplemented with 25 mM of HEPES, 2 mM of glutamine (BioWhittaker, Walkervile, MD), 100 units of penicillin/ml, 100 μg of streptomycin/ml (Gibco, Grand Island, NY), and 10% fetal calf serum (complete RPMI).

**Saliva Collection**

Unstimulated whole saliva samples were collected by “draining.” Each subject sat with his or her head tilted forward so that saliva moved anteriorly in the mouth. After an initial swallow, the subject allowed saliva to drain continuously from the lower lip into a plastic container. At the end of the collection period (5 minutes) the subject expectorated residual saliva.

**Gut Biopsy**

Gut contents and an intestinal biopsy were obtained by using an Entriflex tube with a Carey mucosal biopsy capsule. First the subject’s blood pressure and pulse were measured. Thirty minutes before the tube passage, 10 mg of metoclopramide hydrochloride (Reglan, A. H. Robins, Richmond, VA) was administered orally to help the tube advance rapidly into the small intestine. Immediately preceding tube passage, topical lidocaine was sprayed into the subject’s throat until the gag reflex was fully depressed. The Entriflex tube–Carey capsule was swallowed, and slowly (1 cm/min) advanced through the stomach into the ligament of Trietz. The location of the tube was checked fluoroscopically. To expedite the progression of the tube through the stomach, the subject was at times required to walk or lie on his or her right side or back. The total transit time varied among subjects but was usually less than 1 hour. When the capsule was at the biopsy site, that is, the ligament of Trietz, an intestinal biopsy was obtained by exerting suction pressure with a 50 cc syringe so that the Carey capsule closed slowly. The biopsy was visualized fluoroscopically. After the capsule had closed, suction was maintained while the tube and capsule were gently pulled up and removed from the subject. The specimen was placed in Hanks’ buffered salt solution (HBSS; Gibco) and immediately transported to the laboratory for processing.

**Preparation of Intracellular and Lamina Propria Lymphocytes**

Lymphocytes from the small intestine were prepared from the biopsy specimen (15–17). The washed specimen was treated for 20 minutes at 37°C with 1 mmol/l of dithiothreitol (DTT; Sigma) in calcium- and magnesium-free HBSS containing sodium bicarbonate and HEPES. This was followed by sequential incubations at 37°C with 0.75 mmol/l of ethylenediamine tetra-acetic acid (EDTA) to remove epithelial cells. The cells released by these treatments were pooled. Contaminating enterocytes were removed by magnetic separation using ferrofluid paramagnetic particles (Immunicon, Huntington Valley, PA) coupled with antiepithelial monoclonal antibodies (mAb; Ber EP4, Dako, Carpenteria, CA). For the LPLs to be isolated, the treated tissue was incubated an additional 30 minutes with EDTA and the freed cells were discarded. The remaining tissue was digested at 37°C in 0.01% deoxyribonuclease and 20 U/ml of collagenase (Worthington Scientific, Malvern, PA).

**Immunoglobulin Isotype Concentrations**

Secretory IgA and IgG levels in saliva were measured by enzyme-linked immunosorbent assay (ELISA). Serum IgA, IgG, and IgM levels were measured immunoturbidimetrically with a Cobas Fara centrifugal analyzer (Roche Diagnostic Systems, Inc., Mount Clair, NJ).

**Lymphocyte Proliferation and IL-2 Production**

Cells were cultured in the presence or absence of plate-bound anti-3 mAb (OKT3, CalBioChem, San Diego, CA), plate-bound anti-CD3 + soluble anti-CD28 mAb (CD28.2 IgG1, Pharmingen, San Diego, CA), or plate-bound CD3 + soluble anti-CD28 and anti-CD2 mAb (RPA-2.10 IgG1, Pharmingen). For experiments in which anti-CD3 was used, the mAb was first dissolved in carbonate buffer with a pH of 9.6 at a concentration of 10 μg/ml and then aliquoted into 96-well round bottom tissue culture plates at 50 μl/well. The plates were incubated for 3 hours at 37°C and then washed three times with HBSS to remove unbound anti-CD3. Cells were suspended at 10⁶ cells/ml in complete
RPIM, and 100 µl of cell suspension were added to each well. CD28 and CD2 were added directly to the medium at 10 µg/ml or 2 µg/ml. Plates were then incubated for 48 hours at 37°C in an atmosphere of 5% CO2 and 95% humidity. At 48 hours, 100 µl of cell-free supernatant was removed from each well and replaced with fresh media containing the appropriate treatment. This supernatant was stored at −70°C until analyzed for IL-2. After 68 hours of incubation, 0.5 µCi of [3H]-thymidine in 20 µl was added to each well. At 72 hours of incubation, cells were harvested onto glass microtiter filter paper by using a cell harvester (PHD, Cambridge, MA). The filter disks were placed in 7-ml vials and activity was counted in a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA).

IL-2 levels in cell-free supernatants were measured by an ELISA (Genzyme, Cambridge, MA). Cytokine concentration was determined by comparison to a standard curve generated from serial dilution of recombinant human IL-2 (Genzyme).

Statistical Analysis
The sample size was determined by using the mean and standard deviation obtained from the first 11 subjects. This analysis indicated that n = 17 per age group would be needed to observe a statistically significant difference at p < .05 with 80% power. Immunoglobulin levels were tested for normality, and age differences were determined by using a Student’s t test. Results from proliferation and IL-2 assays were analyzed by using a three-way analysis of variance for the effect of age, treatment, and cell type, as well as their interaction. This was followed for individual comparisons of interest for age and cell type in proliferation and IL-2 production by a Student’s t test, and for the effect of treatment by a paired Student’s t test using SYSTAT software (Evanston, IL). Results are reported as mean ± SEM.

RESULTS

Immunoglobulin Levels
The level of total unstimulated IgA found in whole saliva was 9.97 ± 5 mg/dl for young and 10.4 ± 5 mg/dl for old subjects. Although subject age did not influence salivary IgA levels, serum IgA levels did increase with age (p < .05; see Table 1). Serum levels of IgG and IgM were similar for young and old subjects (Table 1). The secretory IgA level in gastrointestinal fluid collected with the biopsy tissue was below the detection limit of the assay used (data not shown).

Effect of Age on PBMC and Gut-Associated Lymphocyte Proliferation
The analysis of variance indicated a significant effect of age (p < .01), treatment (p < .001), and cell type (p < .007) on lymphocyte proliferation. There was also Age × Treatment (p < .03), Age × Cell type (p < .03), and Treatment × Cell type (p = .04) interaction for lymphocyte proliferation. The PBMCs from both young and old subjects exhibited higher levels of proliferation in response to all four stimulants compared to the media control (p < .05; Figure 1A). Proliferation was similar across treatments. The unstimulated level of proliferation was not affected by subject age. In response to all four treatments, the ability of PBMCs to proliferate significantly diminished with age (p < .05; Figure 1A).

IELs and LPLs from both young and old subjects proliferated in response to all four treatments (p < .05; Figures 1B and 1C, respectively). The response was similar across treatments. Subject age did not affect the unstimulated or stimulated proliferation of IELs, nor did age affect LPL proliferation in response to anti-CD3 alone or in combination with anti-CD28. However, LPLs from young subjects stimulated with anti-CD3 + anti-CD28 + anti-CD2 tended (p < .06 and p < .07, for anti-CD3/CD28/CD2 high and low respectively) to proliferate more than LPLs from old subjects. Proliferation by unstimulated IELs and LPLs was similar, but greater than that of PBMCs of the same age group (p < .05; Figure 1). Proliferation for IELs and LPLs in response to any one of the four treatments was similar and much lower than proliferation by correspondingly stimulated PBMCs, regardless of age (p < .05; Figure 1).

Effect of Age on PBMC and Gut-Associated Lymphocyte Production of IL-2
An analysis of variance indicated a significant effect of age (p = .046), treatment (p = .01), and cell type (p = .01) on IL-2 production. There was also an Age × Treatment (p = .05), Age × Cell type (p = .07), and Treatment × Cell type (p = .08) interaction. The PBMCs from both young and old subjects produced higher levels of IL-2 in response to all four treatments compared to the media control (p < .05; Figure 2A). IL-2 production was similar across treatments. The unstimulated level of IL-2 production was not affected by subject age. The ability of PBMCs to produce IL-2 in response to all four treatments tested decreased significantly with age (p < .05; Figure 2A). Stimulated IL-2 production by IELs or LPLs was similar across treatments (Figures 2B and 2C, respectively). Subject age did not affect unstimulated or stimulated IL-2 production by IELs, but it did affect IL-2 production by LPLs. Stimulated IL-2 production by LPLs from young subjects was significantly higher than that of the older subjects (p < .05). Unstimulated IL-2 production by IELs was similar to that of PBMCs and LPLs of the same age group. Unstimulated IL-2 production by LPLs from young subjects was greater than PBMCs from young subjects. However, there was no difference between LPL and PBMC unstimulated IL-2 production. IELs stimulated with anti-CD3, anti-CD3/CD28, or high anti-CD3/CD28/CD2 produced less IL-2 than similarly treated PBMCs or LPLs regardless of subject age (p < .05). IELs from old subjects stimulated with low anti-CD3/CD28/CD2 produced less IL-2 than did PBMCs, but levels similar to LPLs from old subjects (p < .05). After stimulation with anti-CD3, but not high or low anti-CD3/CD28/CD2 or CD3/
AGE-RELATED CHANGES IN GAIR

CD28, LPLs from young subjects produced higher levels of IL-2 than did PBMCs from young subjects ($p < .05$). LPLs from old subjects produced greater levels of IL-2 than PBMCs from old subjects when stimulated with anti-CD3 or low CD3/CD28/CD2, but not the other two treatments ($p < .05$; Figure 2).

DISCUSSION

This study represents the first analysis of the effect of age on the GAIR in healthy humans who were not undergoing surgery or biopsy for a medical condition and were not subjected to anesthesia. Similar to the response of the peripheral immune system, the GAIR demonstrates age-related changes. The changes in the GAIR were population specific, with age-related changes observed in LPLs, but not in IELs. In the current study, LPLs from old subjects showed significantly lower IL-2 production ($p < .05$) in response to all treatments compared with that of young subjects (Figure 2). Additionally, IL-2 production by PBMCs from old subjects was lower ($p < .05$) than that from the younger subjects, regardless of treatment (Figure 2). Decreased IL-2 production with age by PBMCs from humans and splenocytes from animals has previously been reported, but this is the first report of a decline in IL-2 production with age by LPLs. However, an age-related decline in IL-2 production was speculated to play a role in the age-related impairment of gut mucosal humoral immune responses to mycobacterial antigen (18). Low IL-2 production at intestinal sites may predispose the elderly population to infectious and neoplastic diseases.

In contrast to both LPL and PBMC production, subject age did not influence IEL IL-2 production. This is consistent with the lack of age effect on proliferation of IELs. Furthermore, IELs produced less ($p < .05$) IL-2 upon stimulation than did LPLs and PBMCs. IELs are believed to be less responsive to IL-2 than LPLs are. Additionally, although freshly isolated IELs contain some CD3+ T cells that spontaneously secrete TH1 and TH2 type cytokines, more than 90% of freshly isolated IELs T cells are reported to be in the inactive cell cycle state of G0 to G1 (19). A limited ability to produce IL-2 has been reported when lymphocytes are stimulated with anti-CD3. Alternatively, the lower IL-2 production by IELs could be due to their lower percentage of CD4+ cells. Age-related trends similar to that observed in IL-2 were seen in proliferative response. Although no age-related difference was observed in the proliferative response to different stimuli in IELs, the LPLs from old subjects had a lower proliferative response to high and low anti-CD3/CD28/CD2 ($p = .06$ and $p = .07$, respectively).

Secretory IgA is found in saliva, intestinal secretions, urine, tears, and other mucosal fluids (4). Unlike serum IgA, which is mainly 7S monomers, secretory IgA is an 11S dimer comprising two IgA monomers joined by a covalently linked peptide (J chain). A polypeptide secretory component is also present, which makes sIgA resistant to proteolysis in an enzyme-rich environment. Once secreted, sIgA exhibits a limited ability to fix complement or to promote opsonization, but it actively binds to microorganisms, enterotoxins, and other antigens to prevent adherence and subsequent penetration into the gut wall (4). Therefore, the

Figure 1. Effect of age on the proliferation of A, peripheral blood mononuclear cells; B, intraepithelial lymphocytes; and C, lamina propria lymphocytes. Data are mean ± SEM, n = 17; cpm = counts per minute. Stimulated cultures were significantly different from the media control at $p < .05$ by paired Student’s t test. †Significantly different from the young of the same cell type and treatment at $p < .05$ by Student’s t test; ‡Significantly different from peripheral blood mononuclear cells of the same age group and treatment at $p < .05$ by Student’s t test; †$p < .06$ and $p < .07$ for anti-CD3/CD28/CD2 high and low, respectively, compared with young lamina propria lymphocytes by Student’s t test.
adequate production of sIgA is essential as one of the first lines of protection at mucosal surfaces.

Because of the difficulty in obtaining accurate measurements of immunoglobulin levels in the gut, salivary immunoglobulin levels are often used to make a general representation of the entire mucosal immune system. In the current study, subject age did not influence the level of total, unstimulated sIgA found in whole saliva. This is in concurrence neither with the results reported by Miletic and co-workers (20), which were that salivary sIgA concentration and secretion rate were lower in elderly than in young subjects, nor with those of Arranz and colleagues (21) or Challacombe and coworkers (22), who reported increased salivary IgA in elderly subjects compared with young subjects. Differences in IgA secretion rates and concentration have been attributed to a variety of factors, including a subject’s hydration, diet, daily activity, and level of stress.

In contrast to the salivary sIgA levels, serum IgA levels increased with age in the current study (p < .05; Table 1). Although many studies have reported either no effect or a decline in serum IgA levels with age (22), our results are not without precedent. Smith and colleagues (23) reported a 20-fold higher preimmune serum IgA levels in old (24-month) hamsters than in young (2–3 months). Similar observations have been made in rats and monkeys (24,25). Finkelstein and coworkers (26) and Arranz and colleagues (21) have documented an age-related increase in the serum level of IgA in humans. It is unclear why the salivary sIgA levels were unaffected by age while serum levels increased. Although sIgA is the major immunoglobulin in the gut, IgA is not the predominant isoform in the serum, although it is found at relatively high levels. The function of IgA is still unclear. Subject age did not influence IgG or IgM levels in the serum (Table 1).

In conclusion, the results from the current study demonstrate that IELs, LPLs, and PBMCs are differentially affected by age. LPLs appear to be affected to a greater extent by advancing age than are IELs. However, LPLs appear to be less affected by age than cells in the periphery. The decline in immune function of LPLs, one of the predominant cellular populations in the GAIT, may help explain age-related increases in incidence of infectious and neoplastic disease in the gastrointestinal tract. However, further studies are needed to determine the biological significance of these age-related changes as well as their underlying mechanisms.

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