Immunmodulatorische Wirkungen nicht-essenzieller Lebensmittelinhaltsstoffe - Carotinoide, Alkohol, Pro- und Präbiotika

Habilitationsschrift

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VERZEICHNIS DER ABBILDUNGEN

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CD	Cluster of Differentiation; Oberflächen-Antigen auf Immunzellen
DTH	Delayed-type hypersensitivity; zellvermittelte Immunantwort
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence-Activated Cell-Sorting; Durchflußzytometrie
IFN-γ	Interferon-gamma
Ig	Immunglobulin
IL	Interleukin
LDL	Low-Density-Lipoproteins
LPS	Lipopolysaccharid
MHC	Haupthistokompatibilitätskomplex
NK	natürliche Killer
NRC	National Research Council
PBMC	periphere mononukleäre Blutzellen
PP	Peyer'sche Plaques
PUFA	langkettige, mehrfach ungesättigte Fettsäuren
sIgA	sekretorisches Immunglobulin A
TH	T-Helfer-Lymphozyten
TLR	Toll-like receptors
TNF-α	Tumor-Nekrose-Faktor-alpha

1 Einleitung

Die wichtigste exogene Determinate für den Immunstatus des Menschen ist die Ernährung. Unterernährung bedingt die hohe Infektionsanfälligkeit von Kindern in sogenannten Entwicklungsländern und die damit verbundene hohe Mortalität (Chandra 1972, Scrimshaw et al. 1997). Im Gegensatz dazu sind die Menschen in Industrieländern im allgemeinen ausreichend mit Nahrungsenergie und Nährstoffen versorgt (DGE 2000). Dementsprechend ist eine alimentär bedingte sekundäre Immunschwäche in diesen Ländern selten. Allerdings treten in den Industrieländern zunehmend Probleme mit der Überversorgung an Nahrungsenergie auf, während einzelne Bevölkerungsgruppen wie alte Menschen, Personen mit Essstörungen oder Alkoholiker zeigen Nährstoffdefizite, so dass in diesen Ländern eine alimentär bedingte Immunsuppression zunehmend an Bedeutung gewinnt (Abb. 1).

Abb. 1: Ursachen für alimentär-bedingte sekundäre Immunschwächen

Entwicklungsländer:
Unterernährung
Industrialisierte Länder:
Adipositas
hohe Fettaufnahme
Alkoholismus
Essstörungen (z.B. Anorexia nervosa)
extreme Fehlernährung

Neben den essenziellen Nährstoffen stellen weitere Lebensmittelinhaltsstoffe eine zweite Gruppe von potenziell immunmodulatorisch wirkenden Stoffen dar. Hierzu zählen u.a. einige Vertreter der sekundären Pflanzenstoffe wie Carotinoide und Flavonoide, Alkohol sowie Pro- und Präbiotika. Die Wirkungen dieser Stoffe auf das Immunsystem sind bisher nur unzureichend untersucht.

Weiter gibt es heute fundierte Kenntnisse über die Entstehung zahlreicher Zivilisationskrankheiten und der Beteiligung des Immunsystems an deren Pathogenese. Erste Hinweise aus klinischen Studien deuten auf eine diätetische Beeinflussung des Immunsystems in der Pathogenese dieser Krankheiten hin. Beispiele hierfür sind Herz-Kreislauf-Erkrankungen, Krebs, chronisch-rheumatische Entzündungen und

Lebensmittelallergien. Die Wirkung einzelner Nährstoffe auf die immunologischen Prozesse dieser Krankheiten ist ein wichtiges Forschungsgebiet der Ernährungsimmunologie.

Ein weiterer Schwerpunkt der Ernährungsimmunologie betrifft die Entwicklung funktioneller Lebensmittel mit dem Ziel das Immunsystem „positiv“ zu beeinflussen (enhanced function claim). Im Rahmen des EU-Projektes PASSCLAIM (Process for the Assessment of Scientific Support for Claims on Foods) wurden in der Arbeitsgruppe „Gut Health and Immunity“ hierzu Kriterien erarbeitet, anhand derer die immunmodulatorische Wirkung von Lebensmitteln bzw. spezifischen Inhaltsstoffen überprüft und belegt werden sollte (Cummings et al. 2004). In Zukunft ist von einer vermehrten Entwicklung solcher funktionellen Lebensmittel auszugehen. Diese kurze Einführung in das noch junge Forschungsgebiet der „Ernährungsimmunologie“ zeigt den Rahmen auf, in dem die experimentellen Arbeiten der vorliegenden kumulativen Habilitationsschrift durchgeführt wurden.

1.1 Aufbau des Immunsystems

Das Immunsystem kann in einen unspezifischen (angeborenen) sowie einen spezifischen (erworbenen) Teil untergliedert werden (Abb. 2).

Abb. 2: Der schematische Aufbau des Immunsystems

	unspezifisch	spezifisch
humoral	Komplementsystem	Antikörper
	Lysozym	(Immunglobuline der
	Zytokine	Klassen A, G, M, D, E)
zellulär	Granulozyten	T-Lymphozyten
	Monozyten	(T-Helfer, T-Suppressor)
	NK-Zellen	B-Lymphozyten

Das unspezifische Immunsystem ermöglicht dem Körper, eingedrungene Bakterien und Viren bereits beim Erstkontakt zu eliminieren. Wichtige Zellen hierfür sind die Phagozyten (Monozyten/Makrophagen und neutrophile Granulozyten) sowie die natürlichen Killer (NK)-Zellen. Zusätzlich tragen lösliche Komponenten wie beispielsweise das Lysozym zur unspezifischen Immunabwehr bei.

Das spezifische Immunsystem zeichnet sich durch drei Merkmale aus:

1. Es ist in der Lage, zwischen eigenen und körperfremden Zellen zu unterscheiden;
2. es kann auf spezifische Weise eine Abwehrreaktion gegen eine unbegrenzte Zahl von unterschiedlichen Antigenen ausüben;
3. Bei einem Zweitkontakt mit einem Antigen kann es schnell eine starke Abwehrreaktion auslösen, da es nach dem Erstkontakt mit dem Antigen Gedächtniszellen bildet, die für die Wiedererkennung von Antigenen und für die Verhütung von Reinfektionen bedeutend sind.

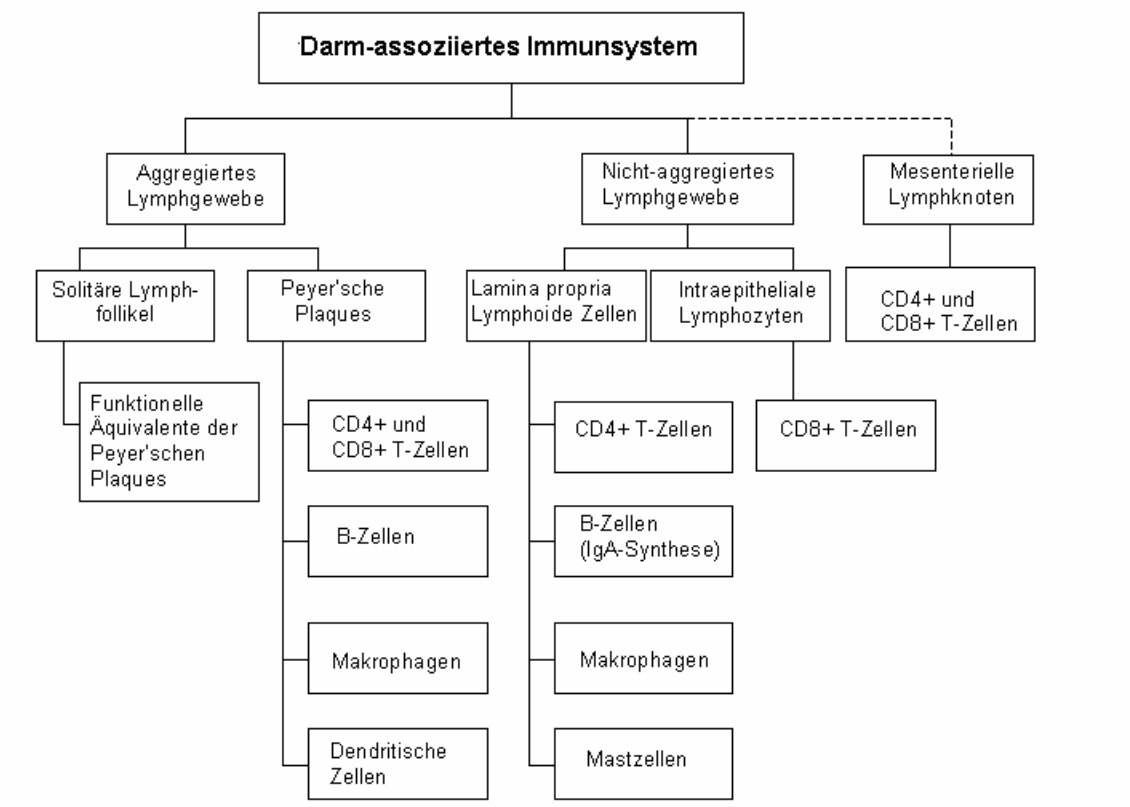
Die hierfür wichtigen Zellen sind die T- und die B-Lymphozyten (Abb. 2). T-Lymphozyten sind für die Regulation der Immunantwort (T-Helferzellen1 und 2 sowie regulatorische T-Lymphozyten) sowie für Unterdrückung unerwünschter Immunreaktionen (T-Suppressorzellen) wichtig. T-Helferzellen modulieren das Immunsystem, indem sie z.B. Zytokine wie das Interferon-γ sezernieren, welche andere Immunzellen aktivieren können. B-Lymphozyten sind für die Antikörperbildung verantwortlich.

Das Immunsystem ist auf verschiedene Kompartimente im Körper verteilt, wobei das Darm-assoziierte Immunsystem das größte Immunorgan darstellt (Mowat 2003) (Abb. 3). Es besteht aus einer großen Anzahl von Immunzellen sowohl in Form von organisierten Strukturen wie z.B. solitäre Lymphfollikel und Peyer'sche Plaques (PP) als auch in Form von vereinzelt zwischen intestinalen Epithelzellen und in der Darmmukosa (Lamina propria) vorliegenden Immunzellen (intraepitheliale Lymphozyten, T-Lymphozyten und dendritische Zellen). Das Darm-assoziierte Immunsystem verhindert, dass krankheitsauslösende Bakterien und Viren das Darmepithel besiedeln oder überwinden und im Körper Infektionen auslösen können. Des weiteren ist es für die sogenannte orale immunologische Toleranz verantwortlich, d. h. es verhindert eine Überreaktion gegen harmlose körperfremde Substanzen, die täglich mit der Darmwand in Berührung kommen (Brandtzaeg 1998).

Für die Auslösung einer Immunantwort im Intestinaltrakt sind primär die Immunzellen der PP wichtig. Die PP kommen überwiegend im terminalem Ileum vor. Im Bereich der PP ist ein spezialisiertes Epithel vorhanden, das sogenannte M-Zellen enthält (Abb. 4). M-Zellen können Antigene (z.B. Makromoleküle, Partikel, Bakterien und Viren) aus dem Darmlumen aufnehmen und zu darunterliegenden Immunzellen transportieren. Dadurch gelangen Antigene in die PP, wo die Antigenpräsentation und damit die Initiation der Immunantwort stattfindet. Dendritische Zellen der Lamina propria sind darüber hinaus in der Lage, die „tight junctions" zwischen den Epithelzellen zu überwinden und aktiv Antigene im Darmlumen zu

binden und in den Bereich der Lamina propria zu transportieren, wo deren immunologische Information aufgearbeitet wird (Nagler-Anderson 2001). Die eigentliche Effektorfunktion im Intestinaltrakt erfolgt durch die Lymphozyten der Lamina propria sowie durch intraepitheliale

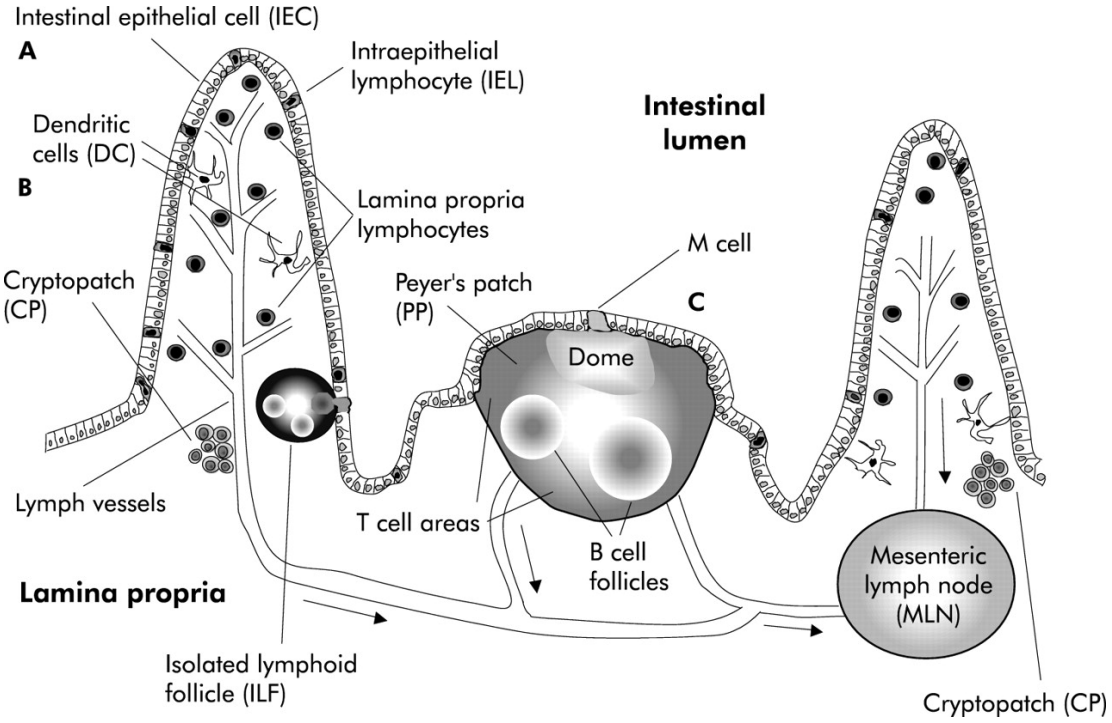
Abb. 3: Das Darm-assoziierte Immunsystem (nach Schley und Field 2002)



Lymphozyten. Beim Erwachsenen finden sich 70-80% aller Ig-produzierenden Plasmazellen des Körpers in der Lamina propria, wobei über 70% der gebildeten Antikörper zur Klasse der IgA-Antikörper zählen (Mowat 2003).

In verschiedenen Studien konnte nachgewiesen werden, dass die intestinale Mikroflora einerseits eine entscheidende Bedeutung für die Entwicklung des intestinalen Immunsystems (z.B. IgA-Produktion) beim Säugling sowie andererseits für die Aufrechterhaltung eines intakten Immunsystems beim Erwachsenen besitzt. Ferner wird vermutet, dass bakterielle Antigene und Lipopolysaccharide aus der Zellwand gram-negativer Bakterien zur normalen Entwicklung und Differenzierung der PP beitragen. Die Entdeckung der „pattern recognition receptors“

Abb. 4: Vorkommen von Immunzellen und lymphoiden Organen im Darm sowie mögliche Aufnahmewege von Antigenen. Luminale Antigene können über (A) intestinale Epithelzellen, (B) dendritische Zellen der Lamina propria und über (C) M-Zellen aufgenommen werden. Über ableitende Lymphgefäße der PP gelangen Antigene in die mesenteriellen Lymphknoten (Spahn und Kucharzik 2004)



wie z.B. den „toll-like-receptors“ (TLR1-10) auf Immun- sowie Epithelzellen hat das Verständnis solcher „pathology-associated molecular patterns“ und ihrer Funktionsweise enorm erweitert (Medzhitov 2001, Abreu 2003). Des weiteren wurde im Tierexperiment nachgewiesen, dass die intestinale Mikroflora für die spezifische Aktivität der natürlichen Killerzellen des systemischen Immunsystems von Bedeutung ist. Keimfreie Mäuse bzw. konventionelle Mäuse mit sterilem Futter zeigten eine niedrige Aktivität der natürlichen Killerzellen (Bartizal et al. 1984). Beim Menschen konnten kürzlich mikrofloraspezifische T-Zellklone in der Peripherie nachgewiesen werden (Duchmann et al. 1999). Dies unterstützt die Hypothese, dass die intestinale Mikroflora eine bedeutende Antigenquelle für den Organismus darstellt.

1.2 Ernährungsimmunologie

Etwa seit den 1960iger Jahren werden die Zusammenhänge zwischen Ernährung und Immunsystem systematisch erforscht (Beisel 1991, Scrimshaw et al. 1997). Das Immunsystem ist ein dynamisches Organ, welches auf eine kontinuierliche antigene Exposition mit Zellproliferation und -differenzierung reagiert und hierfür die ständige Synthese von immunregulatorischen Proteinen und Antikörpern benötigt. Ein Defizit an Makro- und Mikronährstoffen führt zu Beeinträchtigungen der Immunkompetenz verbunden mit einer gesteigerten Infektanfälligkeit (Field et al. 2002). Eine überhöhte Zufuhr einzelner Nährstoffe (z.B. Zink) oder die Qualität der aufgenommenen Fettsäuren beeinflußt ebenfalls die Immunantwort. Deshalb wird die Erfassung des Immunstatus als interessanter Biomarker diskutiert, der zur Bestimmung des Nährstoffbedarfs und/oder der gesundheitlichen Wirkung von Nährstoffen oder Nahrungsergänzungsmitteln eingesetzt werden kann (Bronson et al. 1999). Insgesamt lassen sich die Zusammenhänge zwischen Ernährung und Immunsystem durch 5 zugrundeliegende Wirkebenen charakterisieren (Klasing und Leshchinsky 2000):

1. Regulation des Immunsystems durch Nährstoffverfügbarkeit
2. Regulation des Immunsystems durch spezifische Nährstoffe
3. Indirekte Modulation des Immunsystems durch das endokrine System
4. Modulation der durch die Aktivität des Immunsystems bedingten Schäden
5. Immunsystem-induzierte Änderungen des Nährstoffstatus

Für eine normale Funktion des Immunsystems (Neubildung der Leukozyten, Proliferation, Immunglobulinsynthese, etc.) ist die kontinuierliche Zufuhr adequater Mengen einzelner Nährstoffe als Bausteine (z.B. Aminosäuren) sowie als Co-Faktoren (einzelne Spurenelemente) u.a. für die Proteinsynthese essenziell. Deshalb wirken sich Nährstoffdefizite negativ auf die Entwicklung des Immunsystem sowie die Immunkompetenz aus (Field et al. 2002).
Eine direkte Regulation immunologischer Prozesse erfolgt durch spezifische Nährstoffe wie z.B. den langkettigen mehrfach ungesättigten Fettsäuren (PUFA). PUFA beeinflussen sowohl über den absoluten Gehalt als auch durch das n-3/n-6-Verhältnis die Zellmembranzusammensetzung und damit die Membranfluidität, die interzelluläre Kommunikation sowie die Synthese von Mediatoren wie den Eicosanoiden (Calder und Field 2002). Des weiteren modulieren sie auch die über „toll-like receptors“ ausgelöste Signaltransduktion (Lee et al. 2003).
Das endokrine System reagiert auf die Zufuhr von Nährstoffen durch Bildung von Insulin, Glucagon und Glucocortikoiden, welche immunmodulatorisch wirken. Die hormonelle

Situation bei Adipositas ist ein Grund für die bei Personen mit Fettsucht vorliegende Beeinträchtigung des Immunsystems (Samartin und Chandra 2001). Nährstoffe sind außerdem wichtig für die Begrenzung schädlicher Auswirkungen immunologischer Prozesse wie des oxidativen Burst als Nebeneffekt der Phagozytose. Hierfür sind Antioxidantien wie Vitamin C und E bedeutend. Das Immunsystem kann andererseits direkt über eine Änderung der Nährstoffkonzentration im Blut sowie anderen Körperflüssigkeiten nicht-immunologische Abwehrmechanismen induzieren. Ein Beispiel hierfür ist Eisen, welches als Wachstumsfaktor für bestimmte Bakterien benötigt wird. Während der akuten Phase einer Immunreaktion kommt es zu einer vermehrten Bildung von Eisen- sowie Häm-bindenden Proteinen (z.B. Laktoferrin), wodurch weniger freies Eisen für Pathogene zur Verfügung steht.

Neben einem breiten Spektrum an validierten *in vitro* sowie tierexperimentellen *in vivo*-Methoden zur Erfassung des Immunstatus kann der biologische Effekt einer Immunmodulation auch in klinischen Interventionsstudien untersucht werden. Abbildung 5 enthält eine Gruppe immunologischer Marker, die primär im Rahmen von Interventionsstudien am Menschen zur Bestimmung des Immunstatus eingesetzt werden (Abb. 5). Diese Parameter wurden auch in den eigenen Untersuchungen verwendet.

Abb. 5: Immunologische Marker zur Erfassung einer alimentären Immunmodulation beim Menschen (nach Cummings et al. 2004)

Zell-vermittelte Immunantwort (DTH)	Hauttest
Phagozytenfunktion (Granulozyten, Monozyten)	
A: Phagozytoserate	FACS[1]
B: Generation reaktiver O_2-Verbindungen (Burst)	FACS
Zytotoxische Aktivität der natürlichen Killerzellen	FACS
Zytokinbildung (extra- und intrazelluär)	ELISA, FACS, PCR
Aktivierungsmarker auf Lymphozyten	FACS
Lymphozytenproliferation	ELISA, ^{3}H-Thymidineinbau
Phänotypisierung von Lymphozyten	FACS
Vakzin-spezifische Antikörperbildung	ELISA

[1]: FACS = Durchflußzytometer

2 LEBENSMITTELINHALTSSTOFFE MIT IMMUNMODULIERENDER WIRKUNG

Eine Reihe von Lebensmittelinhaltsstoffen kann nachweislich Immunmechanismen beeinflussen. Hierzu liegen jedoch primär Daten für essenzielle Nährstoffe vor (Calder und Field 2002). Im Gegensatz dazu sind wenig Kenntnisse über die immunmodulatorische Wirkung von nicht-essenziellen Lebensmittelinhaltsstoffen vorhanden. Das Verständnis über das immunmodulatorische Potential sämtlicher Lebensmittelinhaltsstoffe würde Strategien zur Optimierung der Immunkompetenz ermöglichen, und dies sowohl in immunstimulatorischer als auch in immunsuppressiver Hinsicht. Die vorliegende Arbeit fasst den Einfluß von Carotinoiden, Alkohol sowie von Pro- und Präbiotika auf das Immunsystem zusammen.

2.1 Carotinoide

2.1.1 Immunmodulatorische Wirkung der Carotinoide

Gegenwärtig sind etwa 650 Carotinoide bekannt (Watzl und Bub 2001). Im Blut können derzeit etwa 15-20 Carotinoide und etwa 10 Metabolite nachgewiesen werden. Carotine (z.B. β-Carotin und Lykopin) finden sich überwiegend in orange-gelb-rotem Gemüse und Obst, wohingegen Xanthophylle (z.B. Lutein und Zeaxanthin) hauptsächlich in grünblättrigem Gemüse vorkommen. Der Carotinoidgehalt von Gemüse liegt im Durchschnitt um den Faktor 10 höher als im Obst (Watzl und Bub 2001). Auffallend ist die unterschiedliche Hitzestabilität der Carotine und Xanthophylle. Während Carotine eine hohe Hitzestabilität besitzen (Verluste etwa 10%), zeigen Xanthophylle in Abhängigkeit von der Erhitzungsdauer starke Hitzeverluste (> 50%). Das Carotinoidspektrum sowie die Gesamtcarotinoidkonzentration im Plasma (Tab. 1) sind abhängig von den jeweiligen Ernährungsgewohnheiten. β-Carotin stellt bei Erwachsenen in Deutschland das Hauptcarotinoid im Plasma dar (15-30%). Provitamin-A-Wirkung besitzen zwar etwa 50 Carotinoide, aber bei den üblichen Ernähungsgewohnheiten spielen hierfür nur β- und α-Carotin sowie β-Cryptoxanthin eine Rolle.

Carotinoide üben verschiedene physiologische einschließlich immunmodulatorische Wirkungen aus (Watzl und Bub 2001). Bisher ist ausschließlich β-Carotin intensiv am Menschen untersucht worden. Eine Carotinoid-arme Ernährung (< 1 mg/Tag) führte bei jungen Männern, die periodisch einer immunsuppressiv wirkenden UV-

Tab. 1: Konzentrationsbereich für Carotinoide im Plasma

Carotinoid	Konzentration (µmol/L)
β-Carotin	0,04-2,26
α-Carotin	0,02-0,47
Lykopin	0,05-1,40
β-Cryptoxanthin	0,03-0,70
Lutein	0,10-1,30
Zeaxanthin	0,05-0,50

Lichtexposition ausgesetzt waren, zu einer Störung von Immunfunktionen. Die zelluläre Immunantwort gegen intradermal applizierte Antigene (delayed-type hypersensitivity, DTH) war in der Placebo-Gruppe signifikant unterdrückt, wobei die Hemmung invers proportional zur Plasma-β-Carotinkonzentration war. Bei den mit β-Carotin supplementierten Männern (30 mg/Tag) wurde hingegen keine Hemmung der DTH beobachtet (Fuller et al. 1992). Allerdings konnte in anderen Studien kein Einfluß von β-Carotin auf DTH festgestellt werden (Santos et al. 1997).

Bei der Zusammensetzung der Lymphozytensubpopulationen (CD4+, CD4+/CD25+) wurde eine positive Korrelation mit der Plasma-β-Carotinkonzentration beschrieben (Watson et al. 1991, Murata et al. 1994). Eine 2-monatige Supplementierung von β-Carotin (30 oder 60 mg/Tag) erhöhte den Anteil CD3-/CD16+ natürlicher Killerzellen im Blut sowie deren lytische Aktivität (Watson et al. 1991, Prabhala et al. 1991). In neueren humanen Interventionsstudien konnte ebenfalls ein Einfluss von β-Carotin in physiologischen Konzentrationen auf das Immunsystem festgestellt werden. Langzeit-Supplementierung von β-Carotin in einer Dosis von 25 mg/Tag verhinderte bei älteren Männern (65-86 J.) die Alters-bedingte Suppression der Aktivität der natürlichen Killerzellen (Santos et al. 1996), bei jüngeren Männern (51-64 J.) war nach 4 Wochen β-Carotinsupplementierung (15 mg/Tag) die Expression von Adhäsions- und MHCII-Molekülen (ICAM-1, HLA-DR) auf Monozyten sowie die *ex-vivo* Sekretion von Tumor-Nekrosis-Faktor-α erhöht (Hughes et al. 1997).

Von den übrigen Carotinoiden sind beim Menschen bisher nur Lykopin und Lutein in einer einzigen Studie untersucht worden. Im Gegensatz zu β-Carotin war nach oraler Zufuhr von 15 mg Lykopin/Tag bei Monozyten keine Veränderung der Oberflächenrezeptorexpression beobachtet worden (Huges et al. 1997). Die gleiche Dosis Lutein verringerte die Rezeptorexpression signifikant (Hughes et al. 2000). Auffallend ist jedoch, dass die β-Carotinsupplementierung zu einem 5-fachen Anstieg der Plasmakonzentration führte, wohingegen die Lykopinplasmakonzentration nur um 50% gestiegen war (Hughes et al.

2000). Die Lutein-Plasmakonzentration war um das 4-fache erhöht. Somit scheint die Plasmacarotinoidkonzentration in keinem direkten Zusammenhang mit der Rezeptorexpression zu stehen.
In einer prospektiven epidemiologischen Studie im Sudan an 28.753 Kindern wurde der Zusammenhang zwischen der Aufnahme verschiedener Lebensmittel und dem Infektionsrisiko untersucht. Ein hoher Tomatenverzehr war invers mit dem Risiko für Durchfall sowie Infektionen der Atemwege assoziiert, und Husten mit Fieber trat signifikant seltener auf (Fawzi et al. 2000). Diese Ergebnisse deuten auf immunmodulatorisch wirkende spezifische Inhaltsstoffe in Tomaten hin. Hier ist an erster Stelle das Lykopin zu nennen, da die übrigen Tomateninhaltsstoffe auch in anderen pflanzlichen Lebensmitteln häufig vorkommen (Beecher 1998).

Für die immunmodulatorische Wirkung des β-Carotins und anderer Carotinoide ist wahrscheinlich deren antioxidative Wirkung mitverantwortlich (Meydani et al. 1995, Hughes 1999 und 2001, Chew und Park 2004). Das Immunsystem ist ein gegen Oxidationen besonders empfindliches System. Viele Membran-bezogene Prozesse (Eicosanoidsynthese, Rezeptorexpression, interzelluläre Kommunikation) werden durch Oxidation mehrfach ungesättigter Fettsäuren in den Zellmembranen beeinträchtigt. Als Folge treten Störungen der Membranfluidität sowie -integrität auf. Als ein weiterer Wirkmechanismus der Carotinoide wird die Beeinflussung des Signaltransduktionsfaktors NF_KB diskutiert (Meydani et al. 1995). NF_KB ist an der Regulation der Genexpression verschiedener Zytokine sowie von Adhäsionsmolekülen beteiligt (Flohé et al. 1997, Li und Karin 1999). NF_KB wird durch den Redoxstatus der Zelle reguliert und β-Carotin kann den intrazellulären Oxidationsstatus in kultivierten Zelllinien beeinflussen (Palozza et al. 2003). Allerdings lassen sich damit nicht die gegensätzlichen Ergebnisse von β-Carotin und Lutein erklären, da beide antioxidativ wirksam sind (Stahl und Sies 1996). In einer neueren Studie konnte an HeLa-Zellen auch gezeigt werden, dass β-Carotin die Bindung von NF_KB an die DNA erhöhte, was auf eine Induktion oxidativer Veränderungen durch β-Carotin hinweist (Palazzo et al. 2003). Inwieweit Carotinoide - als Bestandteil von Lebensmitteln aufgenommen – zusätzlich Parameter des Immunsystems beim Menschen beeinflussen, wurde in eigenen Interventionsstudien untersucht. Zusätzlich wurde *in vitro* überprüft, ob β-Carotin unter Vermeidung der Umwandlung zu Retinol das Immunsystem stimuliert.

2.1.2 Eigene Untersuchungen

2.1.2.1 *In vitro*-Effekt von β-Carotin auf die Zytokinsekretion (**Publikation 5.1**)

β-Carotin wird vom Menschen enzymatisch in einem gewissen Umfang zu Retinol umgewandelt, so dass in Humanstudien mit β-Carotinsupplementierung nur bedingt zwischen der β-Carotin- und der Retinolwirkung auf das Immunsystem differenziert werden kann. Deshalb wurde in dieser Studie ein *in vitro*-Ansatz zur Untersuchung der Wirkung von β-Carotin auf die Zytokinsynthese gewählt. PBMC von gesunden Probanden wurden isoliert und mit Mitogenen aktiviert. Da β-Carotin über den physiologischen Konzentrationsbereich von 10^{-6} – 10^{-9} µM hinaus konzentrationsabhängig die Zytokinbildung beeinflußt, wurden Konzentrationen bis zu 10^{-11} µM untersucht. β-Carotin hatte keinen Einfluß auf das von TH1-Lymphozyten gebildete Interferon-gamma. Im Gegensatz dazu wurde der Gehalt von Tumor-Nekrose-Faktor-alpha (TNF-α) sowie von Interleukin-1-alpha (IL-1α) im physiologischen Bereich (0,01-1 µM) im Kulturüberstand der PBMC signifikant erhöht. Diese Ergebnisse deuten darauf hin, dass β-Carotin bei den gegebenen Versuchsbedingungen spezifisch die Zytokinsynthese von Monozyten stimuliert. Da kein Einfluß auf die Lymphozyten festgestellt wurde, könnte β-Carotin einerseits zellspezisch wirken, andererseits könnte die Mitogenaktivierung (LPS bei Monozyten, Phytohämagglutinin bei Lymphozyten) sowie der nachfolgende Signalübertragungsweg durch β-Carotin unterschiedlich moduliert werden. Die erhöhte IL-1α–Bildung könnte *in vivo* zur Aktivierung von Lymphozyten beitragen. *In vivo* führte β-Carotin nach Langzeit-Supplementierung (> 2 Monate) tatsächlich zu einer Zunahme an TH-Lymphozyten sowie aktivierten T-Lymphozyten (IL-2R+) (Watson et al. 1991, Prabhala et al. 1991). Die eigenen *in vitro*-Ergebnisse werden zusätzlich durch die Ergebnisse einer Tierstudie bestätigt, in der β-Carotin die TNF-α-Konzentration von Makrophagen, die Tumorgewebe infiltriert hatten, erhöhte (Schwartz et al. 1986). In einer Humanstudie mit 15 mg/Tag β-Carotin wurde ebenfalls eine erhöhte TNF-α-Produktion *ex vivo* nachgewiesen (Hughes et al. 1997). Somit zeigen die eigenen *in vitro*-Versuche, dass β-Carotin als nicht-essenzieller Lebensmittelinhaltsstoff unter diesen Bedingungen einen signifikanten stimulierenden Einfluß auf die Zytokinbildung von Monozyten besitzt, jedoch keine Wirkung auf die Zytokinsekretion von Lymphozyten ausübt.

2.1.2.2 Wirkung von Carotinoid-reichen Lebensmitteln auf das Immunsystem des Menschen (**Publikationen 5.2, 5.3, 5.4, 5.5, 5.6**)

In dieser Interventionsstudie (**Publikation 5.2**) wurde erstmals der Versuchsansatz gewählt, nicht mit reinen Carotinoiden deren Einfluß auf das Immunsystem zu untersuchen, sondern mit Carotinoid-haltigen Lebensmitteln, die einen hohen Gehalt eines bestimmten Carotinoids aufweisen. Hintergrund für diesen Versuchsansatz war die Erkenntnis aus zahlreichen epidemiologischen Studien, dass eine hohe Aufnahme von Carotinoid-reichen Lebensmitteln mit einem verringerten Krebsrisiko korreliert (WCRF 1997). Ein weiteres Merkmal dieser Studie ist eine Carotinoiddepletion während der gesamten Studienphase, in der einzelne Carotinoid-reiche Lebensmittel supplementiert wurden.
Die Intervention führte zu signifikanten Änderungen der Plasmacarotinoidkonzentrationen, womit eine hohe Bioverfügbarkeit für die Carotinoide Lykopin, α- und β-Carotin sowie Lutein aus den jeweiligen Lebensmitteln gezeigt werden konnte. Die gemessenen Immunparameter änderten sich ebenfalls im Laufe der Intervention. Besonders die *ex-vivo* Bildung von IL-2 durch aktivierte PBMC wurde durch die Carotinoid-arme Ernährung gehemmt. Die Supplementierung mit Tomatensaft normalisierte die IL-2-Bildung, wohingegen mit Karottensaft (α- und β-Carotin) und Spinatpulver (Lutein/Zeaxanthin) signifikant weniger IL-2 als zu Beginn der Studie gebildet wurde. Ähnliche, aber weniger stark ausgeprägte Effekte wurden auch für die Parameter IL-4-Bildung sowie Lymphozytenproliferation gemessen. IL-2 ist ein Aktivator der Lymphozytenproliferation. Die unterschiedliche Kinetik der Änderungen beider Parameter deutet darauf hin, dass IL-2 ein empfindlicherer Parameter ist als die Proliferationsantwort. Die Bestimmung des Antioxidantienstatus (**Publikation 5.3**) ergab, dass der Verzehr von Tomatensaft zu einer signifikanten Verbesserung dieses Status führte. Sowohl lag-time als auch die Menge an gebildeten Oxidationsprodukten waren verändert. Dieses Ergebnis bestätigt frühere Arbeiten, die ebenfalls weniger oxidative Schäden nach Verzehr von Tomatenprodukten feststellen konnten (Agarwal und Rao 1998, Rao und Agarwal 1998, Riso et al. 1999).
Insgesamt lassen die Ergebnisse dieser Studie den Schluß zu, dass unter der Voraussetzung einer Carotinoid-armen Ernährungsweise vermehrt oxidativer Stress nachzuweisen ist, der mit einer Beeinträchtigung verschiedener T-Lymphozytenfunktionen einhergeht. Dabei unterscheiden sich die Immuneffekte der einzelnen Interventionsphasen, was möglicherweise auch mit dem unterschiedlichen Carotinoidgehalt der supplementierten Lebensmittel im Zusammenhang steht.

Nachdem in der ersten Interventionsstudie mit 3 verschiedenen Carotinoid-reichen Lebensmitteln besonders für Tomatensaft eine immunmodulierende Wirkung beobachtet

wurde (**Publikation 5.2**), erfolgte die Dürchführung einer weiteren Studie mit ausschließlich Tomatensaftintervention (**Publikation 5.4**). Als Probanden wurden Senioren (Alter 63-86 J.) ausgewählt, da bei diesen im Vergleich zu jüngeren Studienteilnehmern von einer Alters-bedingten Einschränkung der Immunkompetenz auszugehen ist (Lesourd et al. 2002). Die Studienteilnehmer waren in ihrer täglichen Lebensmittelauswahl völlig frei. Die Interventionsdauer wurde in dieser Studie auf 8 Wochen angelegt. Das Spektrum der immunologischen Parameter wurde um die Marker der zytotoxischen Aktivität der natürlichen Killerzellen, die zelluläre Immunantwort (DTH) sowie das Zytokin TNF-α erweitert (Abb. 6).

Abb. 6: Immunologische Parameter der Senioren-Studie

Mitogen-aktivierte Lymphozytenproliferation
Zytokinsekretion (IL-2, IL-4, TNF-α)
Anzahl an CD3-/CD56+-NK-Zellen
Zytotoxische Aktivität der NK-Zellen
Hautreaktion vom verzögerten Typ (DTH)

Obwohl die Plasmakonzentrationen für β-Carotin und Lykopin signifikant zunahmen, war keiner der Immunparameter nach den 8 Wochen im Vergleich zur Kontrollgruppe signifikant verändert. Allerdings korrelierte die Plasma-all-trans-Lykopinkonzentration mit der zytotoxischen Aktivität der natürlichen Killerzellen ($r = 0{,}367$, $p < 0{,}05$); dies kann als Hinweis auf eine immunmodulierende Wirkung des Lykopins interpretiert werden.
Die Ergebnisse einer weiteren Studie mit Senioren, die mit reinem Lykopin (15 mg/Tag) supplementiert wurden, stimmen mit den Ergebnissen unserer Studie überein. Corridan et al. (2001) konnten ebenfalls keine Modulation des Immunsystems feststellen. Die bei den Senioren beider Studien (Watzl et al. 2000, Corridan et al. 2001) gemessenen Plasmakonzentrationen für β-Carotin bzw. Vitamin C lassen den Schluß zu, dass diese Senioren ausreichend Gemüse und Obst verzehrten und der Tomatensaftkonsum bzw. die Lykopinsupplementierung darüber hinaus keine Veränderung des Immun-bzw. Antioxidantienstatus bewirkte. Lediglich bei Senioren mit einem bestimmten genetischen Polymorphismus (R-Allel-Träger der Paraoxonase 1Q192R) war in unserer Studie die LDL-Oxidation nach Tomatensaftkonsum signifikant verringert, nicht jedoch bei dem Gesamtkollektiv (**Publikation 5.5**). Dies deutet wiederum darauf hin, dass eine Änderung des Antioxidantienstatus Voraussetzung für eine Modulation des Immunsystems sein könnte.

Aufbauend auf den Ergebnissen der ersten beiden Interventionsstudien (**Publikationen 5.2 und 5.4**) wurde eine dritte Studie durchgeführt, in der in einem Crossover-Design die Wirkung von Tomaten- und Karottensaft auf das Immunsystem verglichen wurde (**Publikation 5.6**). Wiederum wurde während der gesamten Versuchsdauer eine Carotinoid-arme Ernährungsweise praktiziert. Nach 2-wöchiger Depletion konsumierten die Studienteilnehmer entweder Tomaten- oder Karottensaft (jeweils 330 ml/Tag), worauf eine 2-wöchige Auswaschphase folgte. Danach wurde die Intervention mit den entsprechenden Säften fortgesetzt.

Die Depletion führte zu einer signifikanten Hemmung der TNF-α-Produktion *ex vivo* durch LPS-aktivierte PBMC. Die erste Interventionsphase normalisierte die TNF-α-Produktion und erhöhte signifikant die IL-2-Synthese. Die zytotoxische Aktivität der natürlichen Killerzellen und die Lymphozytenproliferation waren ebenfalls signifikant gesteigert. Zum Ende der ersten Interventionsphase waren sowohl die IL-2-Produktion als auch Lymphozyenproliferation und zytotoxische Aktivität der natürlichen Killerzellen erhöht, was deutlich auf einen Immuneffekt hinweist. Die Erhöhung der TNF-α-Produktion durch Karottensaft bestätigt die *in vitro* gewonnenen Daten zum Einfluß von β-Carotin auf die Bildung dieses Zytokins (**Publikation 5.1**). Bei keinem Parameter wurden jedoch Unterschiede zwischen beiden Säften festgestellt. Auffallend war die zeitliche Verzögerung von einer Woche zwischen den Änderungen der Plasmacarotinoidkonzentrationen und den Änderungen der einzelnen Immunparameter. Interessant wäre es in zukünftigen Studien neben den Plasmakonzentrationen zusätzlich die Carotinoidkonzentration in den PBMC zu messen. Möglicherweise korrelieren Änderungen der intrazellulären Carotinoidkonzentrationen enger mit den Änderungen der Immunparameter als es für die Plasmakonzentration beobachtet wurde.

Wichtig für einen immunmodulatorischen Effekt der Carotinoide scheint eine vorausgehende Carotinoiddepletion zu sein. Unter diesen Voraussetzungen war auch der Antioxidantienstatus durch die Carotinoidsupplementierung verbessert. Beide Studien mit Depletion (**Publikationen 5.2 und 5.6**) konnten einen Carotinoideffekt auf das Immunsystem nachweisen, wohingegen bei uneingeschränkter Ernährungsweise kein Immuneffekt vorhanden war (**Publikation 5.4**). Andere Arbeitsgruppen konnten ebenfalls nur bei Carotinoiddepletion eine Stimulation von Immunfunktionen wie der Lymphozytenproliferation nachweisen (Kramer und Burri 1997, Cross et al. 1998). Daraus läßt sich u.a. eine Bedeutung antioxidativer Mechanismen für die Immunmodulation ableiten.

2.2 Alkohol und Wein

2.2.1 Immunmodulatorische Wirkungen von Alkohol und Wein

Alkoholkonsum kann enorme gesundheitliche Auswirkungen zur Folge haben. So geht ein hoher Alkoholkonsum (> 40 g/Tag) mit einem erhöhten Risiko für verschiedene Krankheiten einher. In Deutschland verursacht ekzessiver Alkoholkonsum von Männern 6.2 % aller Todesfälle (Britton et al. 2003). Moderater Alkoholkonsum (10-30 g/Tag) geht mit einem verringerten Risiko für Herz-Kreislauf-Erkrankungen einher (Renaud et a. 1998, Rimm et al. 1999, Gaziano et al. 1999, Gronbaek et al. 2000). Für Deutschland treten bei Männern dadurch 4,9 % weniger Todesfälle auf als statistisch zu erwarten wären (Britton et al. 2003). Die Alkoholaufnahme beeinflußt auch das Infektionsrisiko. In einer neueren prospektiven Kohortenstudie wurde der Zusammenhang zwischen der Aufnahme an Alkohol, Bier, Schnaps und Wein und dem Auftreten von Erkältungskrankheiten untersucht. Moderater Weinkonsum war invers mit dem Krankheitsrisiko assoziiert, wobei Rotwein wirksamer war als Weißwein (Takkouche et al. 2002). Moderater Alkoholkonsum senkte auch das Infektionsrisiko bei Personen, die mit einem Rhinovirus infiziert wurden (Cohen et al. 1993). Daraus kann ein protektiver Effekt von moderatem Alkoholkonsum auf das Immunsystem abgeleitet werden. Neben der Höhe des konsumierten Alkohols spielt jedoch die gesamte Ernährung eine maßgebliche Rolle für die Auswirkungen des Alkoholkonsums auf das Immunsystem.

Chronischer ekzessiver Alkoholkonsum kann zu starken Schäden der unspezifischen sowie spezifischen Immunantwort führen (Watzl und Watson 1992, Nelson und Kolls 2002), wobei Alkohol (Ethanol) das Immunsystem auf zweierlei Wegen beeinflußen kann. Zum einen hat Alkohol einen direkten toxischen Effekt auf Immunzellen (Abb. 7). Zum anderen beeinträchtigt Alkohol Absorption, Speicherung, endogene Nutzung sowie Ausscheidung von Nährstoffen, was sich zusätzlich zur Toxizität negativ auf das Immunsystem auswirken kann. Die Metabolisierung von Ethanol in der Leber geht mit der Bildung reaktiver Sauerstoffverbindungen einher, die ebenfalls Immunzellen schädigen.

Die direkte Wirkung des Alkohols betrifft die Dosis-abhängige Schädigung der gastrointestinalen Barriere, wodurch es zur vermehrten Aufnahme von Pathogenen bzw. Endotoxinen kommt. Dadurch wird vor allem die Synthese proinflammatorischer Zytokine in der Leber induziert (Watzl und Watson 1993). *In vitro* hemmt jedoch Ethanol die Bildung proinflammatorischer Zytokine durch PBMC (Szabo et al. 1996). Des weiteren wird die Neubildung von Leukozyten im Knochenmark durch Alkohol unterdrückt. Im Tiermodell ist

eine Atrophie von Thymus und Milz zu beobachten mit entsprechenden Änderungen der Lymphozytensubpopulationen. *In vitro* und *in vivo* hemmt Alkohol die Phagozytoserate

Abb. 7: Wirkungen hoher Alkoholkonzentrationen auf das Immunsystem
(↓ = Hemmung, ↑ = Stimulation; G = Granulozyten, M = Monozyten)

- Phagozytose (G, M) ↓
- Oxidativer Burst (M) ↓
- Aktivität der NK-Zellen ↓
- Modulation der Zytokinsynthese/-sekretion (TNF-α ↓, TGF-β ↑)
- Apoptose (Monozyten, PNG) ↑
- Lymphozytenproliferation ↓
- Infektionsresistenz ↓

sowie die damit einhergehende bakterizide Aktivität (Stoltz et al. 1999, Szabo 1999). Zxtotoxische Aktivität der natürlichen Killerzellen und Lymphozytenproliferation nach mitogener Stimulation sind weitere durch Alkohol *in vivo* beeinträchtigte Immunfunktionen (Abb. 7). Allerdings wurde *in vitro* eine Stimulation der natürlichen Killerzell-Aktivität durch Ethanol nachgewiesen (Li et al. 1997). Möglicherweise sind *in vivo* Abbauprodukte des Ethanols wie Acetaldehyd für die suppressiven Immuneffekte mitverantwortlich.

2.2.2 Eigene Untersuchungen

In den eigenen Studien wurde zunächst im Tiermodell die Wirkung von Alkohol auf das Immunsystem (systemisch und lokal im Bereich das Darmes) in Abhängigkeit von der Qualität der Ernährung untersucht. Im Rahmen von Interventionsstudien am Menschen wurde dann der Einfluß von moderatem Alkohol- sowie Rotweinkonsum sowohl akut als auch langfristig auf das Immunsystem erforscht.

2.2.2.1 Einfluß von Alkohol auf das Immunsystem in Abhängigkeit von der Nährstoffzufuhr (**Publikationen 5.7 und 5.8**)

Auf Grund der Wirkung von Alkohol auf die Bioverfügbarkeit sowie die endogene Verwertung, Speicherung und Ausscheidung von Nährstoffen kann die langfristige Alkoholwirkung auf das Immunsystem *in vivo* nur unter Berücksichtigung der jeweiligen Ernährung bewertet werden. Je nach Qualität der Nahrung könnte die immunmodulatorische Wirkung des Alkohols variieren. Deshalb wurde eine Tierstudie durchgeführt, bei der drei verschiedene Futterarten in Kombination mit Ethanol eingesetzt wurden (**Publikation 5.7**). Verwendet wurde eine etablierte flüssige Standardkost (Lieber und DeCarli 1989). Dieses Flüssigfutter ist reich an einfach ungesättigten Fettsäuren (35 % der Gesamtenergie) und enthält u.a. 45-fach mehr Vitamin A und 5-fach mehr Vitamin E als vom National Research Council (NRC 1978) empfohlen. Die Zusammensetzung dieses Futters in Kombination mit Ethanol ermöglichte die Auslösung pathologischer, Alkohol-induzierter Leberveränderung, sie entspricht jedoch nicht der durchschnittlichen Ernährung eines chronischen Alkoholkonsumenten. Im Vergleich zu diesem „Standardfutter“ wurde deshalb ein weiteres flüssiges Futter hergestellt, welches exakt dem Nährstoffbedarf wachsender Ratten entsprach (NRC100) sowie ein Futter mit nur 60 % der empfohlenen Menge an Mikronährstoffen (NRC60).

Die Futterzusammensetzung wirkte sich deutlich auf die immuntoxische Wirkung des Alkohols aus. Während mit der Nährstoff-reichen Standardkost nur geringe Immuneffekte in den Immunorganen Thymus und Milz durch Ethanol festgestellt wurden, traten bei adequater (NRC100) bzw. Mangelernährung (NRC60) besonders starke immunsuppressive Effekte (z.B. Thymusatrophie) nach der Aufnahme von Ethanol auf, die direkt mit dem Nährstoffgehalt korrelierte. Eine durch die Futterqualität modulierte Stimulation von Immunfunktionen durch Ethanol wurde bei der Bildung von IFN-γ, der zytotoxischen Aktivität der natürlichen Killerzellen sowie der Phagozytose gefunden. Futterunabhängig und nur durch Ethanol moduliert wurden die IL-2- und TNF-α-Produktion *ex vivo*.

Insgesamt zeigt diese Arbeit, dass die Futterzusammensetzung einen starken Einfluß auf die immunmodulatorische Wirkung von Ethanol hat. Vermutlich trifft dies auch auf die Situation beim Menschen zu.

Im Bereich des Darm-assoziierten Immunsystems führten die beiden Futterarten NRC100 und NRC60 im Vergleich zum Standardfutter zu einer signifikanten Abnahme an CD4+ T-Lymphozyten sowie sIgA+ Plasmazellen in den Peyer’schen Plaques (**Publikation 5.8**). Somit kann auch das Immunorgan, welche für die orale Toleranz gegen Nahrungsantigene wichtig ist, durch die Kombination beider Faktoren moduliert werden.

2.2.2.2 Akute und Langzeit-Effekte von Alkohol und Rotwein auf das Immunsystem des Menschen (**Publikationen 5.9 und 5.10**)

In epidemiologischen Studien wurde eine erhöhte Infektionsresistenz bei moderatem Alkohol- bzw. Weinkonsum beobachtet, jedoch wurde dieser Zusammenhang bisher in keiner Interventionsstudie bestätigt. Deshalb sollte in zwei humanen Interventionsstudien überprüft werden, inwieweit der akute sowie der tägliche moderate Alkohol- sowie Rotweinkonsum sich auf die Immunkompetenz des gesunden Menschen auswirken. Die Ergebnisse einiger *in vitro*-Studien ließen eine akute Wirkung von Alkohol auf das Immunsystem des Menschen vermuten.

Zunächst wurde der Einfluß eines einmaligen Alkohol- und Rotweinkonsums (500 ml einer 12 % Ethanollösung bzw. von 500 ml Rotwein) auf verschiedene Immunparameter untersucht (**Publikationen 5.9**). Im Gegensatz zu den *in vitro*-Daten konnte jedoch nach einmaligem Alkoholverzehr (12 % Ethanol oder Rotwein) in den nachfolgenden 24 Stunden kein Effekt auf die Phagozytoseaktivität von neutrophilen Granulozyten und Monozyten, Lymphozytenproliferation, zytotoxische Aktivität der natürlichen Killerzellen sowie auf die Zytokinbildung (TNF-α, IL-2, IL-4) festgestellt werden.

Um den potenziell immunsuppressiven Effekt der Rotweinpolyphenole (Middleton et al. 2000) erfassen zu können, wurden als weitere Getränke aus dem selben Traubengut hergestellter entalkoholisierter Rotwein sowie Traubensaft geprüft. Beide polyphenolreichen Getränke waren ohne Einfluß auf das Immunsystem.

Zusätzlich zur akuten Wirkung wurde auch der Effekt einer 2-wöchigen täglichen Aufnahme von 500 ml der jeweiligen Getränke untersucht. Neben den in der Kurzzeitstudie erfassten Parametern wurden die TNF-α mRNA-Expression in den Monozyten, Transforming-Growth-Factor-β im Kulturüberstand aktivierter Monozyten, die Apoptoserate der peripheren Lymphozyten und der Anteil an Vδ2-T-Zellrezeptor-positiven Lymphozyten (Abb. 9) bestimmt (**Publikation 5.10**).

Täglicher Alkoholkonsum in einer Höhe, der das Maß eines moderaten Konsums übersteigt, hatte im Zeitraum von zwei Wochen keine negativen Auswirkungen auf das Immunsystem. Die Zufuhr an Polyphenolen übte ebenfalls keinen meßbaren Einfluß auf das Immunsystem aus. Somit konnten beide Studien übereinstimmend zeigen, dass das Trinken von 500 ml alkoholischer Getränke keinen suppressiven, aber auch keine stimulierenden Wirkungen auf ein breites Spektrum an Immunfunktionen ausübt. Dem zur Folge muß die bei moderatem Alkohol- bzw. Weinkonsum beobachtete erhöhte Infektionsresistenz auf andere Faktoren

Abb. 8: Immunologische Parameter der Langzeit-Rotweinstudie

- Phagozytoserate (%), (G, M)[1]
- Phagozytoseaktivität (mittlere Fluoreszenz) (G, M) [1]
- Zytotoxische Aktivität der NK-Zellen
- Lymphozytenproliferation
- Zytokinsekretion (IL-2, IL-4, TGF-β, TNF-α)
- Zytokin-Genexpression (TNF-α mRNA)
- Apoptoserate (Lymphozyten) (%)
- Anteil der Vδ2-TZR+-Lymphozyten (%)
- Anteil der CD3+-T-Lymphozyten (%)

[1](G = Granulozyten, M = Monozyten)

zurückzuführen sein. Möglicherweise ist moderater Weinkonsum nur ein Indikator für einen gesünderen Lebensstil einschließlich einem erhöhten Verzehr von Gemüse, Obst und Fisch (Gronbaek 2001), wodurch das Infektionsrisiko ebenfalls beeinflußt werden kann.

2.3 Pro- und Präbiotika

2.3.1 Immunmodulatorische Wirkung von Pro- und Präbiotika

Ergebnisse aus tierexperimentellen sowie klinischen Studien lassen einen Einfluß von Probiotika auf das Darm-assoziierte Immunsystem sowie auf das systemische Immunsystem vermuten. Im Gegensatz dazu ist bisher nur wenig über die immunmodulatorische Wirkung der Präbiotika sowie Synbiotika (Kombination von Probiotikum und Präbiotikum) bekannt.

Zahlreiche Probiotikastämme wurden intensiv *in vitro* sowie in Tierexperimenten und Interventionsstudien am Menschen untersucht (Cross 2002, Gill and Cross 2002, Gill 2003). Die Hauptwirkungen der beim Menschen erforschten Probiotika beziehen sich auf eine Stimulation der Phagozytose und der Aktivität der natürlichen Killerzellen. Allerdings wurden häufig keine parallelen Kontrollgruppen in die Studien miteinbezogen, weshalb die Ergebnisse einer weiteren Absicherung bedürfen. Hingegen wurde in einer doppelblinden, Placebo-kontrollierten Studie mit *L. casei* Shirota kein Effekt auf eine Reihe von Immunparametern festgestellt (Spanhaak et al. 1998). Im Gegensatz dazu korrelierte in einem Tiermodell der Kolonkarzinogenese die Aktivierung der natürlichen Killerzellen durch

ein Probiotikum mit einer Hemmung der Tumorbildung (Takagi et al. 2001). Dies gibt einen Hinweis über die biologische Relevanz der Aktivierung der natürlichen Killerzellen.
Vermutlich wirken Probiotika stärker auf das lokale Immunsystem des Darmes als auf das systemische Immunsystem; dies kann jedoch in Humanstudien nicht geklärt werden. Im Tiermodell wurden für verschiedene Probiotika ebenfalls eine Stimulation der Phagozytose, der natürlichen Killerzell-Aktivität sowie der intestinalen sIgA-Produktion gemessen (Gill und Cross 2002). Zudem wurde bei intestinalen Infektions- sowie Tumormodellen eine geringere Mortalität bei den mit Probiotika behandelten Tieren beobachtet.

Lebensmittelinhaltsstoffe, welche die Voraussetzungen für die Klassifikation als Präbiotika erfüllen, dürfen (1) im oberen Gastrointestinaltrakt nicht abbaubar sein, (2) müssen ein spezifisches Substrat für nur eine einzige bzw. eine beschränkte Anzahl an potenziell gesundheitsfördernden kommensalen Bakterien im Kolon sein, und (3) müssen in der Lage sein die Mikroflora im Kolon in Richtung einer gesünderen Zusammensetzung zu verbessern (Gibson und Roberfroid 1995). Häufig in der Lebensmittelproduktion eingesetzte Präbiotika sind Inulin, Oligofructose, Galactooligosaccharide, Lactulose und Lacticol.
Die immunologischen Wirkungen von Präbiotika sind bisher nur unzureichend untersucht (Schley und Field 2002, Watzl et al. 2004). Die postulierte Wirkung von Präbiotika auf das Immunsystem wurden bisher zumeist indirekt aus Tierexperimenten abgeleitet (Gibson und Roberfroid 1995), ohne jedoch direkt den Einfluß auf das Immunsystem zu bestimmen. Präbiotika sollten das Immunsystem stimulieren, da sie die Menge an Bifidobakterien im Dickdarm erhöhen, und die Supplementierung mit Bifidobakterien selbst einen antitumoralen Effekt auslöste (Mizutani und Mitsuoka 1980, Sekine et al. 1985).

In vitro wurde lediglich ein Nigeroseoligosaccharid (eine Mischung von Di-, Tri- und Tetrasacchariden auf Basis der Nigerose [α-(1,3)-Diglucosid]) untersucht. Dieses Oligosaccharid stimulierte die zytotoxische Aktivität von aus Mäuseleber isolierten mononuklären Zellen (Murosaki et al. 2002), woraus ein direkter Effekt dieses Präbiotikums auf Immunzellen abgeleitet werden kann.
In neueren Studien konnte mit verschiedenen Präbiotika alleine oder in Kombination mit einem Probiotikum ein immunmodulatorischer Effekt im Tiermodell festgestellt werden. Vorrangig wurde eine Stimulation der sIgA-Bildung in Ileum und Caecum sowie eine Aktivierung der Phagozytose und der natürlichen Killerzellen beobachtet (Watzl et al. 2004). Besonders häufig wurden Veränderungen von Immunmechanismen bei Zellen aus den Peyer'schen Plaques gemessen. Möglicherweise wirken Fermentationsprodukte der Präbiotika primär im Bereich des Darm-assoziierten Immunsystems.

Aus Humanstudien liegen bisher nur aus einer Studie Daten zur immunologischen Wirksamkeit vor (Guigoz et al. 2002). In dieser Studie wurde eine Hemmung der Phagozytoseaktivität von Granulozyten und Makrophagen durch Fructooligosaccharide beobachtet. Zusätzlich war die mRNA-Expression von IL-6 verringert, was als anti-inflammatorischer Effekt interpretiert wurde. In weiteren vier Humanstudien wurden Präbiotika ausschließlich in Kombination mit Probiotika oder Nahrungsergänzungsmitteln geprüft, wobei jedoch Effekte nicht auf die Einzelkomponenten zurückgeführt werden können. Insgesamt ist festzuhalten, dass die potenziellen immunologischen Wirkungen von Probiotika, Präbiotika und Synbiotika beim Menschen wenig untersucht und vor allem hinsichtlich der Wirkung auf das Darm-assoziierte Immunsystem methodisch gegenwärtig nur unzureichend zu erfassen sind.

2.3.2 Eigene Untersuchungen

2.3.2.1 Kurzzeit-Effekte von Pro-, Prä- und Synbiotikum auf das Immunsystem der Ratte (**Publikation 5.11**)

Ziel dieser Studie war es, nach vierwöchiger Fütterung die Wirkungen von Probiotika, Prä- und Synbiotikum auf das systemische sowie Darm-assoziierte Immunsystem der Ratte zu untersuchen. Als Probiotika wurden *Lactobacillus rhamnosus* GG und *Bifidobacterium lactis* BB12 verabreicht, als Präbiotikum mit Oligofructose angereichertes Inulin. Um die Vergleichbarkeit der Ernährung der Ratte mit der des Menschen zu verbessern, wurde ein fettreiches, ballaststoffarmes Rattenfutter verwendet. Der absolute Fettgehalt des Futters alleine beeinflußt bereits das Immunsystem (Barone et al. 1989) und möglicherweise dadurch auch das immunmodulatorische Potenzial von Pro- und Präbiotika.

Immunparameter wurden im Blut, in der Milz sowie in den Peyer'schen Plaques und den mesenteriellen Lymphknoten bestimmt. Prä- und Synbiotikumbehandlung führten zu einer Stimulation der sIgA-Produktion im Caecum bzw. im terminalem Ileum sowie der IL-10-Produktion durch Zellen der Peyer'schen Plaques. Im Gegensatz zu den Daten der Literatur wurde kein Effekt auf die Aktivität der natürlichen Killerzellen und der Phagozyten beobachtet. Insgesamt wurde primär eine Beeinflußung der Immunzellen der Peyer'schen Plaques sowie der intestinalen sIgA-Produktion festgestellt und nur geringe systemische Effekte. Auffallend war auch die stärkere immunmodulatorische Wirkung des Prä- bzw. Synbiotikums im Vergleich zum Probiotikum.

2.3.2.2 Langzeit-Effekte von Pro-, Prä- und Synbiotikum auf das Immunsystem der Ratte sowie auf die Entstehung von AOM-induziertem Dickdarmkrebs (**Publikation 5.12**)

In dieser Langzeitstudie wurde der Effekt auf das Darm-assoziierte Immunsystem sowie auf die Kolonkarzinogenese untersucht. Ein Teil der Ratten wurde mit dem Karzinogen Azoxymethan behandelt, wodurch die Entstehung von Dickdarmkrebs ausgelöst wird. Über die Dauer von 33 Wochen wurden alle Tiere mit den gleichen Pro-, Prä- und Synbiotika behandelt wie in der Kurzzeitstudie. Die Synbiotikum-Behandlung verringerte die Anzahl an Adenoma und Karzinoma im Dickdarm im Vergleich zu den mit Kontrollfutter behandelten Tieren (Femia et al. 2002). Gleichzeitig war die Aktivität der natürlichen Killerzellen in den Peyer'schen Plaques signifikant erhöht. Dies könnte bedeuten, dass die Aktivität dieser Zellen für die Erkennung und Elimination der intestinalen Tumore eine Rolle spielt. Zusätzlich könnte die zytotoxische Aktivität weiterer Immunzellen des Darmes (z.B. intraepithelialen Lymphozyten), die in dieser Studie nicht untersucht wurden, ebenfalls stimuliert sein. Bei den nicht mit dem Karzinogen behandelten Ratten erhöhte die Präbiotikum-Behandlung die Killerzell-Aktivität der Milzzellen. Analog zum Kurzzeitversuch war auch nach 33 Wochen eine erhöhte IL-10-Produktion *ex vivo* sowohl durch die Zellen der Peyer'schen Plaques als auch der mesenteriellen Lymphknoten festzustellen. Die vermehrte IL-10-Bildung kann als ein Mechanismus interpretiert werden, der einerseits für die Regulation der Aktivität der natürlichen Killerzellen bedeutend ist, und andererseits für die Kontrolle inflammatorischer Prozesse. Die Probiotika-Behandlung führte zu einer verringerten IFN-γ-Produktion durch Zellen der Peyer'schen Plaques. Fundierte Daten zum protektiven Mechanismus der Synbiotikum- bzw- Präbiotikum-Behandlung liegen jedoch nicht vor. Ein möglicher Mechanismus könnte die vermehrte Bildung von kurzkettigen Fettsäuren sein, wie sie im Darm der mit Prä- und Synbiotikum-behandelten Ratten quantifiziert wurden (Femia et al. 2002). Für Butyrat konnte *in vitro* eine suppressive Wirkung auf die Zytokinproduktion von TH1-Lymphozyten und eine Steigerung der IL-10-Synthese nachgewiesen werden (Säemann et al. 2000, Cavaglieri et al. 2003). In neuen Arbeiten wurden erstmals zwei spezifische G-Protein gekoppelte Rezeptoren (GPR43 und GPR41) für kurzkettige Fettsäuren auf verschiedenen Immunzellen identifiziert (Le Poul et al. 2003, Brown et al. 2003). Ob über diese Rezeptoren die Zytokinsynthese durch Fermentationsprodukte der Präbiotika moduliert werden kann, muß in weiteren Studien geklärt werden.

3 ZUSAMMENFASSUNG

Ziel dieser Arbeit war zu untersuchen, ob nicht-essenzielle Lebensmittelinhaltsstoffe das Immunsystem beeinflussen. Die Besonderheit der vorliegenden Arbeit liegt in der Prüfung von kompletten Lebensmitteln (Carotinoid-reiche Säfte, Rotwein) im Gegensatz zu isolierten Inhaltsstoffen. Darüber hinaus wurden die immunmodulatorischen Effekte primär im Rahmen humaner Interventionsstudien durchgeführt. Aus den Ergebnissen dieser Studien geht klar hervor, dass solche Lebensmittelinhaltsstoffe immunmodulatorisch wirksam sind. Für diese nicht-essenziellen Lebensmittelinhaltsstoffe kann gegenwärtig jedoch die Frage nicht beantwortet werden, welche Zufuhrmengen für eine optimale Immunfunktion benötigt werden.

Die Ergebnisse der Untersuchungen zu den Carotinoiden zeigen, dass die alimentäre Zufuhr von Carotinoiden als natürlicher Bestandteil von Lebensmitteln immunmodulatorisch wirksam ist. Eine Carotinoid-arme Ernährungsweise führt zu einer Unterdrückung verschiedener Immunfunktionen. Im Gegensatz dazu stimuliert der Verzehr Carotinoid-reicher Säfte als Supplement zur Carotinoid-armen Basisernährung die Zytokinsynthese (IL-2, TNF-α) sowie die Aktivität der natürlichen Killerzellen. Da eine hohe lytische Aktivität der natürlichen Killerzellen mit einem verringerten Krebsrisiko einhergeht (Imai et al. 2000), könnte dieser Zusammenhang für das bei Carotinoid-reicher Ernährung beobachte geringere Krebsrisiko (WCRF 1997) mitverantwortlich sein.

Alkoholkonsum kann das Risiko für verschiedene Krankheiten einschließlich Infektionskrankheiten sowohl erhöhen als auch senken. In einer Tierstudie mit unterschiedlicher ernährungsphysiologischer Futterqualität konnte eine Beeinflußung der Alkohol-induzierten Immunmodulation gezeigt werden. Eine nicht-bedarfsgerechte Ernährung verstärkte die immuntoxische Wirkung von Ethanol. Dem zur Folge ist auch beim Menschen mit Alkoholkonsum von einem starken Einfluß der Ernährung auf das Alkohol-bedingte Krankheitsrisiko auszugehen. In zwei Humanstudien wurde der Effekt eines akuten bzw. chronischen, moderaten Alkoholverzehrs auf das systemische Immunsystem untersucht. Dabei wurden weder Kurzzeit- (24 Stunden) noch Langzeiteffekte (2 Wochen) der Alkoholaufnahme auf das Immunsystem festgestellt. Somit ist bei moderatem Alkoholkonsum, unabhängig von der Form (Rotwein oder 12 % Ethanollösung) nicht von einer signifikanten immunmodulatorischen Wirkung auszugehen.

Schließlich wurde die Wirkung von Pro- und Präbiotika auf das Darm-assoziierte Immunsystem im Tiermodell untersucht. Die Kurzzeit-Behandlung mit einem Probiotikum hatte nur geringe Auswirkungen auf das Immunsystem, wohingegen das Präbiotikum alleine oder in Kombination mit dem Probiotikum die intestinale sIgA-Bildung sowie die IL-10-Bildung ausschließlich durch Zellen der Peyer'schen Plaques stimulierte. Besonders bedeutend ist das Ergebnis der Langzeitstudie mit Prä- und Synbiotikum-supplementierten Tieren, die vorher mit einem Dickdarmkarzinogen behandelt wurden. Bei diesen Tieren waren verschiedene Funktionen von Immunzellen der Peyer'schen Plaques aktiviert und gleichzeitig traten signifikant weniger Tumore im Kolon auf. Hervorzuheben ist hier die zytotoxische Aktivität der natürlichen Killerzellen, die u.a. für die Erkennung und Elimination von Tumorzellen wichtig sind (Smyth et al. 2001). Insgesamt waren unter den gegebenen Voraussetzungen dieser Tierstudien (z.B. fettreiche, ballaststoffarme Ernährung) das Präbiotikum sowie das Synbiotikum stärker wirksam als das Probiotikum alleine. Des weiteren scheint durch diese Behandlung in erster Linie das Darm-assoziierte Immunsystem moduliert zu werden.

Zusammenfassend zeigen die Ergebnisse der vorliegenden Arbeit, dass auch nicht-essenzielle Lebensmittelinhaltsstoffe das Immunsystem beeinflussen. Zukünftige Empfehlungen zur optimalen Förderung des Immunsystems sollten deshalb neben den essenziellen Nährstoffen auch sekundäre Pflanzenstoffe sowie Pro- und Präbiotika berücksichtigen.

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BETA-CAROTENE IN VITRO STIMULATES TUMOR NECROSIS FACTOR ALPHA AND INTERLEUKIN 1 ALPHA SECRETION BY HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

Beta-carotene (BC) potentially affects cancer resistance by stimulating secretion of immunoregulatory cytokines and thereby modulating immune defenses. Therefore, we investigated the effects of BC applied in vitro on the secretion of interleukin-1 alpha (IL-1), tumor necrosis factor alpha (IL-1), tumor necrosis factor alpha (TNF) and interferon-gamma (IFN) by human peripheral blood mononuclear cells (PBMC). PBMC from healthy individuals were activated with pokeweed mitogen (PWM; 0.1 ug/ml), or lipopolysaccharide (LPS; 10 ug/ml) for 24 hr, or phytohemagglutinin (PHA; 5 ug/ml) for 74 hr. BC was encapsulated in liposomes and delivered to PBMC during mitogen activation at concentrations of 10^{-6} to 10^{-11} M. The concentration of cytokines in the supernatants of activated cells was measured by ELISA. BC had no impact on IFN release. The release of IL-1 was significantly ($p < 0.05$) stimulated by BC (10^{-6} to 10^{-8} M). No effect on IL-1 secretion was obtained when PBMC were incubated with BC-free liposomes. However, BC-free liposomes suppressed significantly TNF secretion (from 1.5 to 0.2 ng/ml). When BC was presented in liposomes at concentrations ranging from 10^{-6} to 10^{-8} M it stimulated significantly ($p < 0.01$) TNF secretion. We suggest that physiologically achievable concentration so BC stimulate monokine secretion. Such changes might explain in part the observed immunomodulatory effect of BC on lymphoid cells and reduced cancer risk associated with high intake of BC.

Key words: Interleukin, Beta-carotene, Tumor Necrosis factor.

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INTRODUCTION

Carotenoids are common constituents in green, leafy vegetables and many colored fruits. Beta-carotene (BC) is a carotenoid of special interest as it possesses activity in cancer prevention. Epidemiological studies show a positive correlation between a high dietary intake of BC and prevention of cancer (1,2). Human prospective and retrospective studies demonstrated that BC may be protective against lung and stomach cancer, and possibly against cancer of ovary and cervix, but not against colon or rectal cancer (3). One possible mechanism by which BC exerts its cancer preventive activity could be due to immunostimulation (4-13).

In addition to the accumulation of BC in plasma and tissues, it is partially converted into retinol by the intestinal enzyme beta-carotenoid 15, 15'-dioxygenase. Although both retinol and BC can function in cancer prevention and immunomodulation, a recent review suggests that the BC molecule plays a role which is independent of its conversion into retinol (10). BC has been associated with regression of hamster buccal pouch carcinoma concomitantly with induction of TNF (tumor necrosis factor alpha) secretion by macrophages (8). This suggests a role of cytokines in immunostimulation and immunoregulation by BC. In animals anti-tumor activities of BC (12) were correlated with immunocompetence at the cellular level in which the cytokines act as essential transmitters of cell to cell information. BC incorporated in liposomes increased in vitro the tumoricidal activity of LPS stimulated human monocytes (13). Since the maximum tumoricidal activity was observed after 24 hr of incubation, TNF could have mediated this activity increase. Preliminary data have shown that the administrations of very high levels of BC for a short period of time increased one type of lymphocyte subset in humans (14). In vitro incubation of lymphocytes with carotenoids by not with retinoids, significantly activated natural killer cells, and physiological concentrations of BC increased the numbers of PBMC with markers for activations (14). Because BC in vitro is not converted into retinol, we decided to use the in vitro system in order to identify BC effects without retinol production and involvement. In this study we report the in vitro effects of BC on the secretion of immunoregulatory cytokines by human PBMC which might accomplish the reported anti-cancer activities of BC.

SUBJECTS AND METHODS

Subjects Eleven healthy volunteers (age: 41 $\pm$ 11 years) with no history of medically documented illness, nor use of nutrient supplements or medications participated in this study. The study was approved by the Human Subjects Committee of the University of Arizona.

Cell Culture Conditions Human peripheral blood mononuclear cells (PBMC) were collected in heparinized blood vacationers and separated on Ficoll Hypaque (Organon Teknika) as described elsewhere (4). Briefly, the blood was diluted 1:1 with phosphate-buffered saline (PBS) and was layered on top of the lymphocyte separation medium. After centrifugation at 360xg for 15 min PBMC were collected and washed twice with PBS. Cells were then adjusted to a concentration of 1×10^6 cells/ml and re-suspended in complete RPMI-1640 media (supplemented with 10% FCS, 2 mM L-

Glutamine, penicillin and streptomycin). The cells ($1x10^5$/well) were activated with a T-cell mitogen, PHA (Sigma Chemical Co., St. Louis, MO) at 5 ug/ml, a T-independent B-cell mitogen, PWM (Sigma Chemical Co.) 0.1 ug/ml, or LPS (E. coli 0111: B4; Difco, Detroit, MI) 10 ug/ml. In addition, the mitogen-activated cells were simultaneously incubated with either BC encapsulated in liposomes or liposomes alone. BC encapsulated in liposomes was serially diluted from 10^{-6} to 10^{-11}M. BC-free liposomes containing the same amount of phosphlipids were diluted in the same way.

PWM and LPS-activated leukocytes were incubated in a humidified 5% CO_2 incubator at 37°C for 24 hr and PHA-stimulated cells for 72 hr. At the end of the incubation period, plates were centrifuged for 10 min. at 180 xg and supernatants were collected and frozen at -20°C.

Cytokine Assays Measurement of cytokines in the supernatants of cultured PBMC was done by using ELISA systems as described previously (15). Briefly, 96 well flat-bottom plates (Immulon-II, Dynatech Inc., McLean, VA) were coated overnight with murine anti-human monoclonal antibodied. These antibodies were specific for human IL-1 (clone C42), TNF (clone F12), and IFN (clone A07), all were obtained from Olympus, Lake Success, NY. Between subsequent steps in the assay, coated plates were washed twice with PBS containing 0.05% Tween 20. Plates were incubated with supernatants and cytokine standards for 1 hr at room temperature. Then, polyclonal antibodies, rabbit anti-human antibodies specific for IL-1, TNF, or IFN were added and incubated for 1 hr. After washing, affinity purified peroxidase conjugated goat anti-rabbit IgG antibodies (American Qualex, La Miarada, CA) were added. After incubation and washing, the peroxidase substrate, 2,2'-Azino-bis-3-Ethylbenzothiazoline 6-sulphonic acid (Sigma Chemical Co.) was added. The quantity of each cytokine was determined by comparison of the optical density measured at 405 nm with a set of standards generated by recombinant human IL-1, TNF or IFN (all obtained from Genzyme, Boston, MA).

Preparation of Liposomes BC was dissolved in chloroform in short neck round-bottom flasks with the mixture of each 5mg of phospholipids: phosphatidylcholine from fresh egg yolk (Sigma), L-phosphatidylserine (Sigma) from bovine brain, and L-phosphatidylethanolamine (L-lecithin) from egg (Avanti Polar Lipids, Pelham, AL). Multilamellar vesicles (MLV) were prepared by rotatory evaporator protected from light under argon at 46°C. After 5 times of freezing-thawing the MLV were extruded by extruder (Nucleopore, Pleasanton, CA) in a nitrogen atmosphere through 0.1mm PC membrane filter (Nucleopore) for 10 times filtration in order to obtain unique size of unilamellar vesicles (liposomes). The concentration of BC encapsulated in liposomes was determined by HPLC. Control liposomes without BC were prepared in the same manner as described above. The size of liposomes was measured by a 5mW He/Ne laser machine with 632.8nm wave length (Brookhaven Instruments Corporation BIC), Holtsville, NY, and by laser light scattering goniometer (BIC) (16-18).

Statistical Analysis Analysis was done using ANOVA tests and 2-tail student's t-test. The level of significance was defined at p 0.05.

RESULTS

Effect if BC on Interferon-Gamma (IFN) Secretion No significant difference were observed in IFN production by mitogen stimulated cells treated either BC incorporated in liposomes or BC-free liposomes after 72 hr incubation (Fig.1). The data were expressed as a percentage of the media control 100% equal to 3.1 ng/ml of IFN. The cells stimulated with mitogens only without liposomes or liposomes + BC.

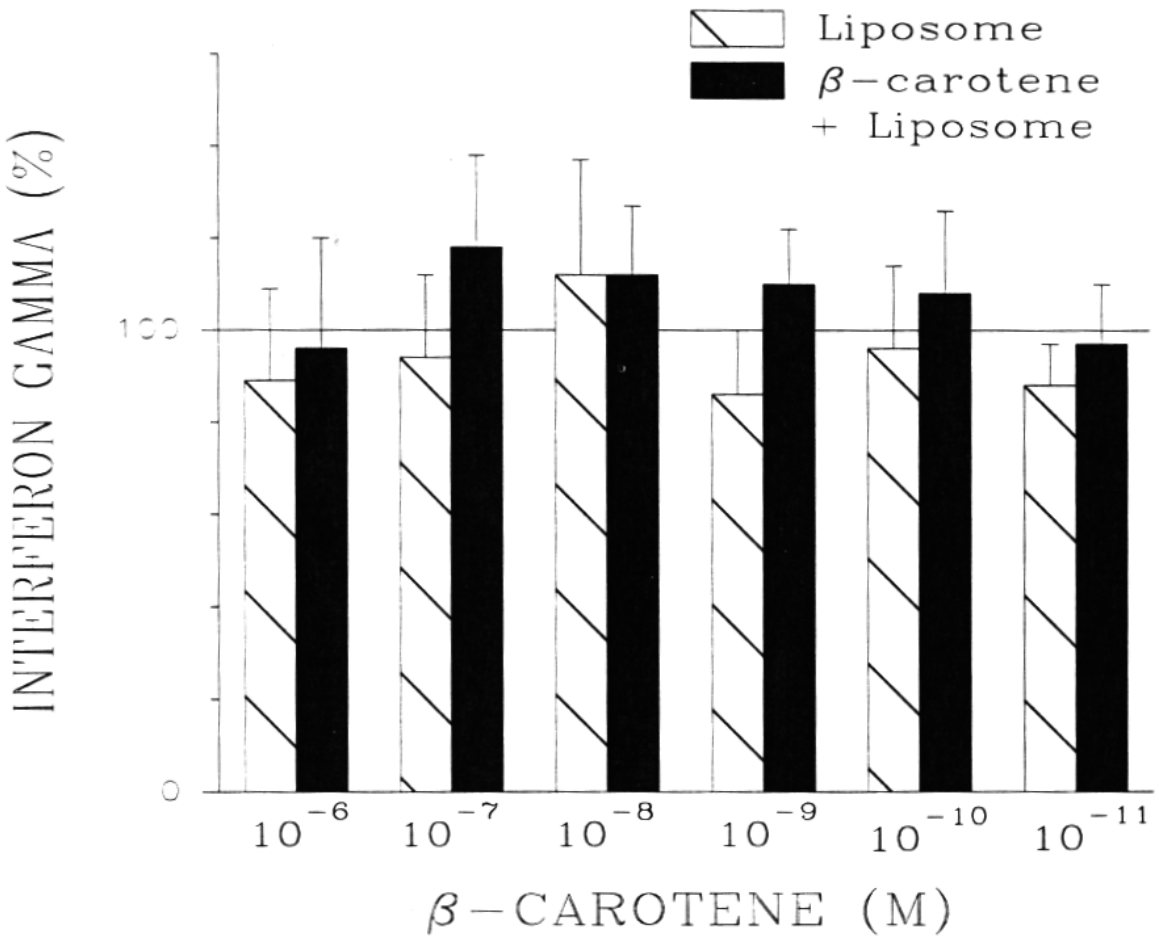

FIG. 1. Effect of BC on IFN secretion by PBMC in vitro. Liposomes with and without BC were prepared with the same amount of phospholipids and the same volume of liposomes preparation was added to PBMC. Cells were stimulated for 72 hr with 5 ug/ml PHA. Concentration of IFN was measured by ELISA. * = $P<0.05$, ** $P<0.01$. The dotted line represents the media control (cells stimulated with mitogens).

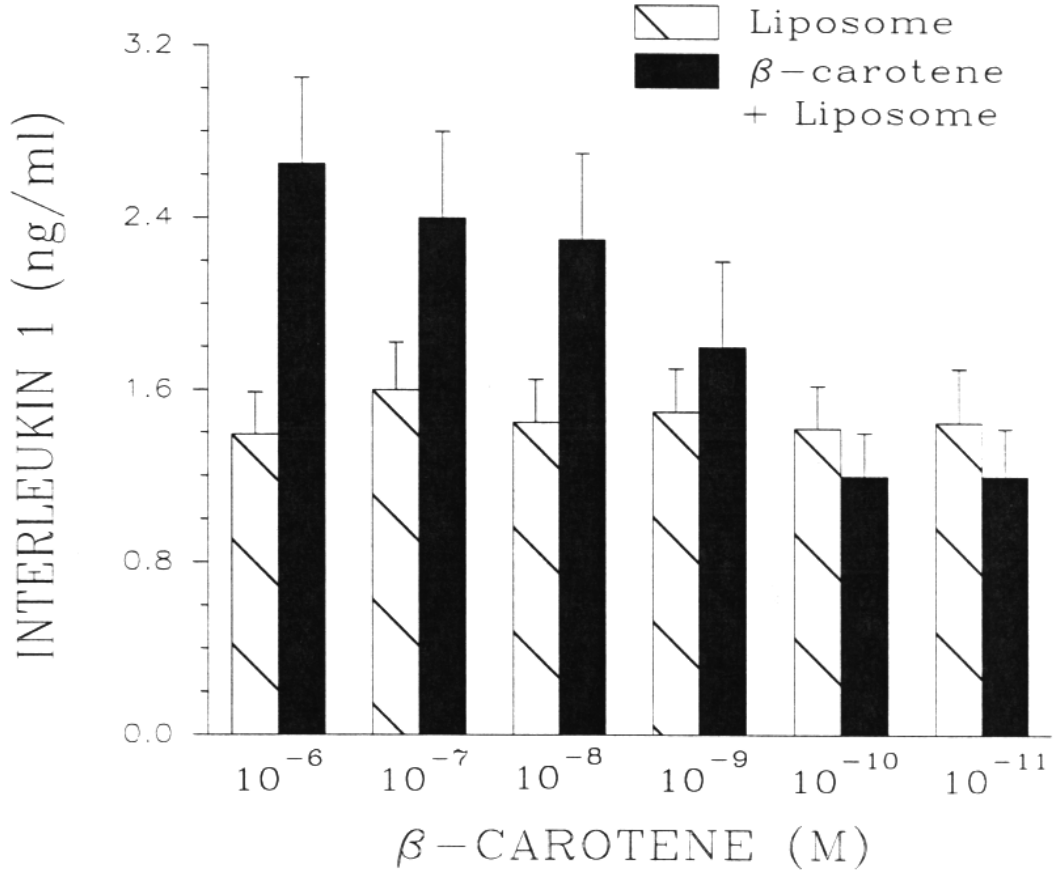

Fig. 2. Effect of BC on IL-1 secretion by PBMC in vitro. Liposomes with and without BC were prepared with the same amount of phospholipids and the same volume of liposomes preparation was added to PBMC. Cells were stimulated for 24 hr with 0.1 ug/ml PWM. Concentration of IL-1 was measured by ELISA. * $p < 0.05$, ** $p < 0.01$, the dotted line represents media control (cells stimulated with PWM)

Effect of BC on Interleukin-1 Alpha (IL-1) Secretion: BC-treated cells showed a dose-dependent stimulation of IL-1 release (Fig. 2). Liposomes containing BC producing final concentrations of 10^{-6}M to 10^{-8}M induced IL-1 secretion which was significantly higher ($p < 0.05$) than those of cells treated with BC-free liposomes. IL-1 secretion by stimulated PBMC cultured with encapsulated BC (1×10^{-6}M) increased from 1.4 to 2.65 ng/ml. BC-free liposomes did not modulate significantly the IL-1 secretion of activated PBMC.

Effect of BC on Tumor Necrosis Factor Alpha (TNF) Secretion: As shown in Fig. 3, the culture of LPS-activated PBMC with liposomes without BC significantly suppressed TNF secretion ($p < 0.05$) compared with activated cells cultured in medium without liposomes. Incubation of PBMC with BC encapsulated in liposomes not only prevented the suppressive effects of liposomes, is highly significantly ($p < 0.01$) stimulated TNF secretion in a dose dependent manner (Fig. 3).

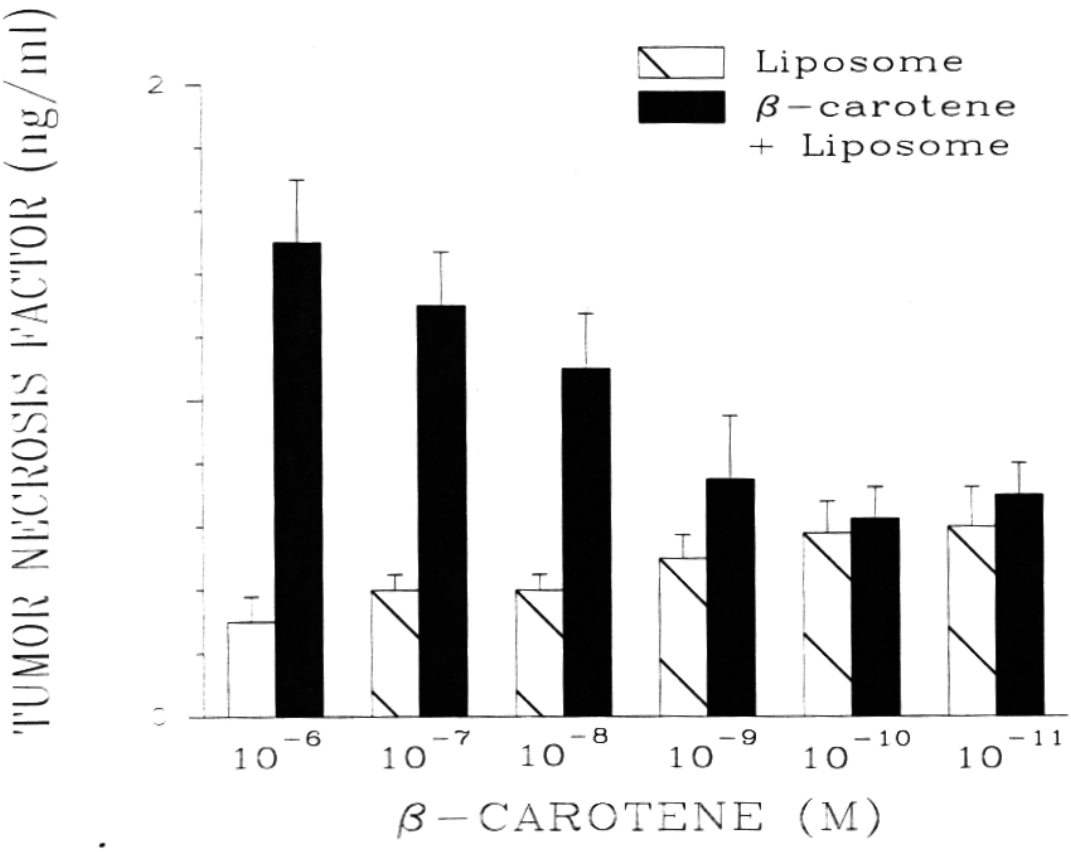

Fig. 3. Effect of BC on TNF secretion by PBMC. Liposomes with and without BC were prepared with the same amount of phospholipids and the same volume of liposomes preparation was added to PBMC. Cells wee stimulated for 24 hr with 10 ug/ml LPS. Concentration of TNF was measured by ELISA. * $p < 0.05$, ** $p < 0.01$. The dotted line represents the media control (cells stimulated with LPS).

DISCUSSION

Both BC and metabolite, retinol, have been associated with a reduced risk of certain types of cancer. This has been suggested to be due in part to immunostimulation (5). Immunomodulatory effects of BC appear independent of its provitamin A activity (4,5,19). By using an in vitro model we were able to evaluate the direct effects of BC on cytokine secretion by PBMC. In vitro systems allow investigation of the direct effects of BC on lymphoid cells without interference with liver metabolism or the influence of the neuroendocrine system. Our results show that BC affected the secretion of monocyte-derived cytokines. Physiological concentrations of BC increased the secretion of IL-1 and TNF, while that of lymphokine-derived IFN was not affected. These in vitro results may help to interpret the mechanisms behind the in vivo immunomodulating effects of BC.

The significant increase in IL-1 release by PBMC induced by BC could be a mediator of the activation of T-helper cells during in vitro (5,14) or in vivo (4,5) BC treatment. These studies showed an increase in the number of T-helper cells and activated lymphocytes (IL-2R$^+$ cells) after 2 or 3 months (4,5,7), but not after 1 month of BC consumption (20). Short-term, in vitro treatment with BC but not retinol (14) increased the number of activated lymphocytes expressing surface IL-2 receptors.

The enhanced release of IL-1 by BC administration may explain the increased mitogenesis of B lymphocytes upon mitogen stimulation which was reported by Bendich et al. (19). Recent reports showed elevated numbers of natural killer (NK) cells ($CD16^+$) after BC treatment (4,5). There was significant increase in the percentage of PBMC activity after treatment with BC (4,5). IL-1 is able to stimulate the cytotoxicity of NK cells and the enhanced secretion of IL-1 by PBMC may potentiate their cytotoxicity. IL-1 is an important signal for T-helper cell activation. Mitogens are capable to induce T helper cells to enter the G1 phase from the resting GO phase of the cell cycle. IL-1 can initiate T cells to enter the S phase of the cell cycle and induce the production of IL-2. BC may play a role in the quantitative increase of activated immune cells (4,5) as well as a potent activator of IL-2 secretion. However, after 72 hr incubation with BC the secretion of IFN by PBMC was not affected.

Schwartz et al. (8,9) administered BC to hamsters which resulted in the reduction of chemically-induced tumor growth. BC concomitantly increased the numbers of macrophages that were stained positively for TNF in the tumor bearing tissue. We also demonstrated that BC stimulated monocytes to produce novel proteins with tumoricidal activity (6). Liposomes interact with cell membranes via endocytosis or fusion of vesicles or in exchange of vesicles with cell membrane lipids (16-18). Our observation of increased TNF secretion by PBMC treated with encapsulated BC may explain the report from Moriguchi et al. (13) that indicated in vitro activation of tumorcidial capacities of human monocytes by BC encapsulated in liposomes. These reports thus support our finding that BC activates monocytes to release more TNF than cells which were not exposed to BC. This stimulated secretion of TNF from monocytes was not caused by an increase of IFN release, as the addition of BC to PBMC had no effect on the secretion of IFN.

Liposomes without BC suppressed the LPS-induced TNF secretion when compared to LPS-activated cells without liposomes exposure. This phenomenon might be explained by the findings of a recent report (22). LPS incorporated in liposomes diminished the tumoricidal activity and the TNF secretion by murine macrophages up to 100 fold. The incorporation of LPS in liposomes masked the lipid A moiety of the LPS and thereby abrogated the interaction between macrophages and LPS (21). Such an effect may have occurred in our experiment, in which LPS and liposomes were added separately, but at the same time to PBMC. This may explain the suppression of TNF release caused by liposomes in the presence of LPS. The other mitogens used in this study obviously did not interfere with the liposomes, since we did not observe any difference in cytokine secretion between mitogen-activated PBMC with and without liposomes.

It is known that oxidative damage decreases macrophage membrane receptors, which are important for an antigen presentation, and that BC together with canthaxanthin protected these receptors from oxygen damage (22). BC could also protect membrane-bound TNF and IL-1 from oxygen damage and result in higher concentrations of released cytokines. Recently it was shown that retinoids are required for the release of TNF (23), which suggests further interactions between retinoids, carotenoids, and cytokine secretion.

Our results show that BC in vitro has immunostimulatory effect on monocular

cells by increasing their secretion of the immunoregulatory cytokines IL-1 and TNF. This study may help to understand the hypothesis which suggested that BC has potential anti-cancer activity as it is able to enhance the secretion of immunoregulatory cytokines.

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Modulation of human T-lymphocyte functions by the consumption of carotenoid-rich vegetables

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A human intervention study was conducted to determine the effect of the consumption of carotenoid-rich vegetables on the immune system. Subjects, (twenty-three men), who were non-smokers, were not restricted in their daily diet, except that they had to abstain from fruit and vegetables high in carotenoids throughout the whole study period. The study was divided into four periods, each lasting 2 weeks: weeks 1–2: low-carotenoid period; throughout weeks 3–8: daily consumption of 330 ml tomato juice (40 mg lycopene/d, 1·5 mg β-carotene/d) (weeks 3–4), 330 ml carrot juice (21·6 mg β-carotene/d, 15·7 mg α-carotene/d, 0·5 mg lutein/d) (weeks 5–6), 10 g dried spinach powder (11·3 mg lutein/d, 3·1 mg β-carotene/d) (weeks 7–8). Blood was collected weekly from subjects after a 12 h fast. T-lymphocyte functions were assessed by measuring proliferation and secretion of immunoreactive cytokines. The consumption of a low-carotenoid diet resulted in a significantly reduced proliferation of peripheral blood mononuclear cells (PBMC) cultured with concanavalin A. After 2 weeks of tomato juice consumption and until the end of the intervention period lymphocyte proliferation was not significantly changed compared with proliferation at the end of the depletion period. Secretion of cytokines by T-helper-1-like lymphocytes (interleukin (IL)-2) and by T-helper-2-like lymphocytes (IL-4) was influenced by the dietary intervention. IL-2 and IL-4 secretion values were significantly suppressed after the low-carotenoid diet ($P < 0{\cdot}001$ and $P < 0{\cdot}05$ respectively compared with baseline). Tomato juice consumption significantly enhanced IL-2 ($P < 0{\cdot}001$) and IL-4 secretion ($P < 0{\cdot}05$) compared with the end of depletion period. After carrot juice and spinach powder consumption the cytokine secretion capacity of PBMC was not significantly different from that at the end of the depletion period. In conclusion, the results of the present study indicate that a low-carotenoid diet reduces T-lymphocyte functions and addition of tomato juice restores these functions. This modulation could not be explained by changes in the plasma carotenoid concentrations. The active constituents in tomato juice as well as the biological significance of this immunomodulation remain to be determined.

Carotenoids: Vegetable consumption: T-lymphocytes: Cytokines

Carotenoids are common constituents in many vegetable and fruit varieties (Mangels *et al*. 1993). Only for β-carotene, with its provitamin A activity, have the biological activities of carotenoids been intensively studied (Burri, 1997). With the results of the Alpha-Tocopherol Beta-Carotene (ATBC) and Carotenoid Retinol (CARET) studies (The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994; Omenn *et al*. 1996), however, interest has increased in the physiological effects of carotenoids other than β-carotene. Recently, lycopene and oxocarotenoids (xanthophylls) have become a major focus in carotenoid research (Stahl & Sies, 1996; Bone *et al*. 1997; Gerster, 1997).

The immunomodulatory activity of β-carotene has been thoroughly investigated in several animal species and in a number of human trials (Bendich, 1989; Watson *et al*. 1991; Meydani *et al*. 1995*a*). While the animal studies have shown an immunoenhancing effect of pure β-carotene (Bendich, 1991), results from human trials are inconsistent. Studies have reported stimulation of various immune functions with β-carotene supplementation (Meydani *et al*. 1995*b*; Moriguchi *et al*. 1996; Santos *et al*. 1996; Hughes *et al*. 1997*a*,*b*; Kazi *et al*. 1997) or have observed no effects (Meydani *et al*. 1995*b*; Kramer & Burri, 1997). Most human studies have used pure β-carotene in dosages ranging from 15 to 300 mg/d for short- and long-term periods (Meydani *et al*. 1995*b*).

Abbreviations: ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; ConA, concanavalin A; IL, interleukin; PBMC, peripheral blood mononuclear cells.

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Depletion studies with low-β-carotene diets have revealed conflicting results. Daudu *et al.* (1994) reported no effect on lymphocyte proliferation of a low-β-carotene diet consumed for 68 d, while Kramer & Burri (1997) observed a suppressed proliferative responsiveness of lymphocytes in human subjects consuming a low-β-carotene diet (0·5 mg/d) for 60 d. These controversial results may be due to methodological differences in the protocol of peripheral blood mononuclear cell (PBMC) isolation.

Besides β-carotene only lycopene has been studied in human subjects. Supplementation with 15 mg lycopene/d for 28 d induced no consistent changes in monocyte surface marker expression (Hughes *et al.* 1997*b*). The immunomodulatory activity of other carotenoids in human subjects is not known. There is one study which investigated the effects of consuming carotenoid-rich vegetable extracts on lymphocyte functions (T-lymphocyte proliferation) in human subjects (Clevidence *et al.* 1997). In this study a carotenoid complex from vegetables was consumed, which provided β-carotene (3·3 mg/d), lycopene (0·6 mg/d) and lutein/zeaxanthin (1·5 mg/d). Lymphocyte proliferation was enhanced after the consumption of the carotenoid complex. So far, no study has investigated the effects of consuming carotenoid-rich vegetables on the human immune system.

The objective of the present study was to examine the effects of consuming different carotenoid-rich vegetable products on T-lymphocyte functions in healthy adult male subjects. Carotenoids (α-carotene, β-carotene, lycopene, lutein) were provided by highly accessible food matrices (processed vegetable products) in amounts possibly consumed by people eating a Mediterranean-type diet. In a preliminary study we showed that for processed carotenoid-rich vegetables a supplementation period of 2 weeks was sufficient to increase plasma carotenoid concentrations 2–3-fold (H Müller, A Bub, B Watzl and G Rechkemmer, unpublished results). In the present study, after a carotenoid depletion period of 2 weeks the low-carotenoid diet of the subjects was supplemented daily for 2 weeks with tomato juice (lycopene) followed by carrot juice (α-carotene, β-carotene) and by spinach powder (lutein). No wash-out periods between the different supplementation periods were used in order to mimic more closely the dietary behaviour of consumers. Plasma concentrations of major carotenoids (including *trans*- and *cis*-isomers and epoxides), α- and γ-tocopherols, retinol, and ascorbic acid were measured (Müller *et al.* 1999). T-lymphocyte functions were assessed by measuring mitogen-activated proliferation. In order to detect whether T-lymphocytes were affected as a total cell population or whether subgroups of T-lymphocytes were differentially affected, the secretion of T-helper-1 lymphocyte specific (interleukin (IL)-2) and T-helper-2 lymphocyte specific (IL-4) cytokines was quantitated.

Subjects and methods

Subjects

Twenty-three non-smoking men (aged 27–40 years), with normal weight (BMI 23·1 (SD 0·39) kg/m^2) were recruited for the study. All subjects were in good medical health as determined by a screening history and medical examination. None was taking vitamin supplements or medications from 1 month before or during the study. The study was approved by the Medical Ethical Committee of the Landesärztekammer Baden-Württemberg and all participants gave their consent in writing. All participants were employees from the Research Centre Karlsruhe, where the Institute of Nutritional Physiology of the Federal Research Centre for Nutrition is located.

Study design

This study was conducted during the months of October to December. The study was divided into four periods each lasting 2 weeks, resulting in a total study period of 8 weeks: weeks 1–2 low-carotenoid period, weeks 3–4 tomato juice consumption (330 ml/d providing 40 mg lycopene and 1·5 mg β-carotene; Schoenenberger, Magstadt, Germany), weeks 5–6 carrot juice consumption (330 ml/d providing 21·6 mg β-carotene, 15·7 mg α-carotene and 0·5 mg lutein; Schoenenberger), weeks 7–8 spinach powder consumption (10 g/d providing 11·3 mg lutein and 3·1 mg β-carotene; Völpel, Königsmoos, Germany). In order to supply a standardized quantity of carotenoids through the vegetables, we used vegetable juices. Since there was no juice from green, lutein-rich vegetables available on the German market, we decided to use spinach powder, which was dissolved by the study subjects in water, milk, yoghurt or soup. Subjects were told to consume the vegetable products with their main meals. Subjects were not restricted in their daily diet except that they had to abstain from fruit and vegetables high in carotenoids throughout the whole study period. A list of the fruit and vegetables the subjects were not allowed to eat was provided (Müller *et al.* 1999). Subjects had to record their daily fruit and vegetable consumption throughout the whole study period. The daily carotenoid intake provided by fruit and vegetables was quantified by using a dietary assessment software package (PRODI 4.4, Nutri-Science, Karlsruhe, Germany), which is based on the German Food Code and Nutrition Data Base (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, 1994). Total carotenoid and ascorbic acid intakes of the study subjects before the study period were assessed using a validated food-frequency questionnaire (Boeing *et al.* 1997).

Preparation of peripheral blood mononuclear cells

Blood from fasting subjects was collected once weekly between 07.00 and 09.00 hours; plasma was separated and an equal amount of PBS was added. PBMC were isolated by density-gradient centrifugation using Histopaque 1077 (Sigma, Deisenhofen, Germany) and were washed twice with PBS. Purified PBMC were resuspended in complete RPMI-1640 culture medium (Life Sciences, Eggenstein-Leopoldshafen, Germany), containing 50 ml fetal bovine serum/l (Life Sciences), L-glutamine (2 mmol/l), penicillin (100 000 U/l) and streptomycin (100 mg/l). Trypan blue staining was used to assess cell viability, and lymphocytes were counted in a haemocytometer under a light microscope.

Leucocyte numbers

Total leucocyte numbers were determined on fasting blood after each week. Leucocyte numbers were obtained by using an automated analyser (F-300, Sysmex, Hamburg, Germany) according to standard operational procedures.

Lymphocyte proliferation

PBMC from each subject at 1×10^9 cells/l in complete RPMI-1640 medium were cultivated in quadruplicate in flat-bottomed ninety-six-well microtitre plates (Greiner, Nürtingen, Germany) and stimulated by the T-cell mitogen concanavalin A (ConA; 5 mg/l; Sigma) for 120 h at 37° in an atmosphere of 50 ml CO_2/l and 95 % humidity. PBMC were pulse-labelled with the thymidine analogue 5-bromo-deoxyuridine (100 μmol/l; Boehringer, Mannheim, Germany) for 3 h at 37°. The incorporated 5-bromo-deoxyuridine was detected by a quantitative cellular enzyme immunoassay using a commercial ELISA kit (Boehringer), following the manufacturer's instructions. The amount of incorporated 5-bromo-deoxyuridine is quantified by measuring the absorbance of the samples in a multiplate spectrophotometer (Molecular Devices, Menlo Park, CA, USA) at 450 nm (reference wavelength 650 nm). As background control absorbance was measured in wells which were not pulse-labelled with 5-bromo-deoxyuridine. Proliferative responses were expressed as the net absorbance values (absorbance$_{A450\,nm - A650\,nm}$ of pulse-labelled cells − absorbance$_{A450\,nm - A650\,nm}$ of unlabelled cells).

Interleukin-2 and interleukin-4

PBMC from each subject at 1×10^9 cells/l in complete RPMI-1640 medium were stimulated in quadriplicate by 5 mg ConA/l for 48 h at 37°. Microplates were centrifuged for 5 min at 300 ***g*** and cell-free supernatant fractions were collected and stored at −80° until analysis. IL-2 and IL-4 were quantified by ELISA methods. For IL-2, microtitre plates (Maxisorp, NUNC, Roskilde, Denmark) were coated with 50 μl of an anti-human IL-2 monoclonal antibody (4 mg/l carbonate buffer, pH 9·6; R&D Systems, Wiesbaden, Germany) and stored overnight at 4°. Between subsequent steps in the assay, plates were washed five times with PBS-Tween (0·01 M-PBS containing 0·5 ml Tween 20/l) with an automated plate washer (Molecular Devices). Non-specific binding was blocked by adding 50 μl bovine serum albumin (10 ml/l in PBS-Tween; Boehringer) for 1 h at 37°. Plates were incubated with supernatant fractions and with serial dilutions of recombinant human IL-2 (R&D Systems) diluted in complete RPMI-1640 medium. Then 50 μl of a polyclonal goat anti-human IL-2 neutralizing antibody (8 mg/l PBS-Tween and 10 ml bovine serum albumin/l; R&D Systems) was added for 1 h at room temperature, followed by a further 1 h of incubation with 50 μl of a rabbit anti-goat immunoglobulin G peroxidase-conjugated antibody (dilution 1 : 40 000 in PBS-Tween and 10 ml bovine serum albumin/l; Jackson ImmunoResearch Laboratories, Hamburg, Germany). As a peroxidase substrate, 100 μl of a 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid buffer (ABTS; Sigma) (0·54 g ABTS and 21 g citric acid monohydrate, in 1 litre water, pH 4·2, with final concentration of 0·3 ml H_2O_2/l) was used. Optical density was measured with a multiplate spectrophotometer (Molecular Devices) at 405 nm.

IL-4 was measured in a similar way to IL-2 with the following reagents. Coating: polyclonal anti-human IL-4 (0·5 mg/l; Endogen, Cambridge, MA, USA); blocking: 40 ml bovine serum albumin/l in PBS-Tween; standards: recombinant human IL-4 (Endogen); detecting antibody: biotin-labelled anti-human IL-4 monoclonal antibody (0·25 mg/l; Endogen); peroxidase-conjugated streptavidin (0·5 mg/l; Jackson ImmunoResearch Laboratories).

Statistics

The results for plasma carotenoids and leucocyte numbers are expressed as means and standard deviations and the results for the immunological measurements as means with their standard errors. Differences between values for week 2 (end of depletion) and supplementation with the vegetable products for 2 weeks within the same group were examined by Wilcoxon matched pairs signed rank test. Statistical significance was accepted at $P < 0{\cdot}05$. All statistical calculations were performed with the Statistical Analysis Systems computer software program (SAS for Windows, version 6.12; SAS Institute, Cary, NC, USA).

Results

Plasma carotenoids

Compliance of the study subjects was high, with no subject missing the daily intake of the vegetable products. During the 2-week low-carotenoid period there was no statistically significant change in plasma carotenoids compared with prestudy plasma concentrations (Table 1). Tomato juice, carrot juice, and spinach powder consumption each resulted in a significant increase of the vegetable-specific carotenoids compared with plasma concentrations at the end of the depletion period (Table 1). The increase in lycopene concentration in week 4 was not associated with changes in other carotenoids (Müller *et al*. 1999). In week 6 plasma lycopene concentration returned to baseline concentration. Plasma retinol, α-tocopherol and ascorbic acid concentrations did not change throughout the intervention period (Müller *et al*. 1999; A Bub, B Watzl, J Wever, SL Abrahamse, H Delincée, ST Adam, S Mittenzwei, J Hegele, H Müller and G Rechkemmer, unpublished results). The average daily intake of total carotenoids from self-selected foods during the study period based on food records was 1·32 (SD 0·57) mg, with 57 % derived from β-carotene, 6 % from α-carotene, 36 % from lutein and 2 % from lycopene. Total carotenoid and ascorbic acid intakes before the study period were calculated to be 3·48 (SD 2·17) mg/d and 114·3 (SD 48·9) mg/d respectively.

Leucocyte numbers

Total leucocyte numbers were constant throughout the whole study period starting with $5{\cdot}5 \times 10^9$ cells/l and ending with $5{\cdot}2 \times 10^9$ cells/l (range $5{\cdot}1$–$5{\cdot}5 \times 10^9$ cells/l).

Table 1. Plasma carotenoid concentrations (μmol/l) and proliferation and interleukin-4 production (pg/ml) by peripheral blood mononuclear cells of subjects consuming a low-carotenoid diet (weeks 0–2) supplemented with tomato juice (weeks 3–4), carrot juice (weeks 5–6) or spinach powder (weeks 7–8)†

(Mean values and standard deviations (plasma carotenoids) or standard errors (lymphocyte functions) for twenty-three subjects)

	Baseline		Depletion			Tomato juice				Carrot juice				Spinach powder			
Week...	0		1	2		3		4		5		6		7		8	
	Mean	SD or SE	Mean	SE	Mean	SD or SE	Mean	SE	Mean	SD or SE	Mean	SE	Mean	SD or SE	Mean	SE	Mean	SD or SE
Plasma‡																		
Lycopene	0·16	0·07	–		0·16	0·08	–		0·38*	0·13	–		0·15	0·05	–		0·14	0·06
β-Carotene	0·74	0·44	–		0·60	0·36	–		0·65	0·25	–		2·05*	0·72	–		1·21*	0·51
Lutein	0·37	0·14	–		0·35	0·12	–		0·33	0·12	–		0·36	0·11	–		0·71*	0·17
Lymphocyte																		
Proliferation§	1·57	0·06	1·61	0·08	1·47	0·08	1·25*	0·05	1·52	0·07	1·60	0·06	1·61	0·06	1·53	0·06	1·31	0·06
Interleukin-4	35·0*	5·3	29·6	14·6	27·9	3·1	26·5	16·5	36·4*	4·0	33·3*	4·4	30·8	3·0	32·5	4·3	26·9	3·6

Mean values were significantly different from those for week 2: *$P < 0·05$.
† For details of diets and procedures, see pp. 384–385.
‡ Data are from Müller *et al.* (1999). Plasma carotenoids were not determined at weeks 1, 3, 5 and 7.
§ Measured as absorbance at 450 nm minus absorbance at 650 nm.

Lymphocyte functions

In a dose–response curve with ConA, 5 mg ConA/l gave the maximum proliferative responsiveness and maximum cytokine secretion. This concentration was, therefore, used throughout the study. Lymphocyte proliferation decreased at the end of the low-carotenoid period (week 1 *v.* week 2, $P = 0·07$) and after the first week of tomato juice consumption proliferation was significantly suppressed compared with proliferation at the end of the depletion period (Table 1). After a further week of tomato juice consumption and until the end of the intervention period proliferation did not differ compared with proliferation at the end of the depletion period (Table 1).

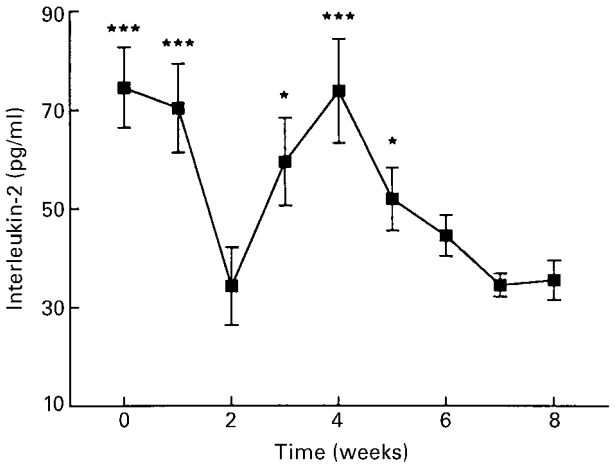

Fig. 1. Concentration of interleukin-2 (pg/ml) secreted by peripheral blood mononuclear cells (PBMC) activated with concanavalin A (5 mg/l) and cultured for 48 h at 37° and 50 ml CO_2/l. PBMC were isolated from subjects (*n* 23) who consumed a low-carotenoid diet (weeks 0–2), which was supplemented with tomato juice (weeks 3–4), carrot juice (weeks 5–6) or spinach powder (weeks 7–8). Values are means with their standard errors represented by vertical bars. Mean values were significantly different from those for week 2: *$P < 0·05$, ***$P < 0·001$.

The concentrations of immunoreactive IL-2 secreted into the culture media by the PBMC from study subjects when cultured with ConA are shown in Fig. 1. There was a significant decline in the capacity to secrete IL-2 after 2 weeks of consuming the low-carotenoid diet. The supplementation with tomato juice activated PBMC to secrete IL-2 in an amount similar to the prestudy concentration. After the 2 weeks of carrot juice consumption and after two further weeks of spinach powder consumption no increased capacity to secrete IL-2 compared with week 2 was observed.

The capacity of PBMC to secrete immunoreactive IL-4 was also modulated by the dietary intervention. The low carotenoid intake during the first 2 weeks resulted in a significant reduction of IL-4 secretion (Table 1). Tomato juice consumption for 2 weeks increased IL-4 secretion capacity of PBMC significantly. Again, 2 weeks of carrot juice consumption and of spinach powder consumption did not result in an enhanced IL-4 secretion compared with the end of the depletion period.

Discussion

The immune system is a major component in the pathogenesis of several chronic diseases like cancer and cardiovascular disease. Epidemiological studies have consistently found that an inverse relationship between intake of vegetables and fruit and risk for these diseases exists (Steinmetz & Potter, 1991; Block *et al.* 1992; Key *et al.* 1996; Ness & Powles, 1997). The constituents in vegetables and fruit contributing to the preventive effects are not yet identified. In addition, a strong inverse association between dietary intake of β-carotene and cancer risk has been observed in several epidemiological studies (Mayne, 1996). Since dietary β-carotene is mostly provided by vegetables and fruit, the immunomodulatory activity of β-carotene may contribute to the observed protective effects of these food items.

Subjects enrolled in our present study had a total carotenoid intake of 3·48 (SD 2·17) mg/d before entering the study. Since the average carotenoid intake of males in

Germany (aged 24–50 years) is 5·6 mg/d (Pelz *et al*. 1998), our study subjects were consuming relatively low levels of fruit and vegetables. The results of our study show that the consumption of a diet low or high in carotenoid-rich vegetable products modulates T-lymphocyte functions as measured in *in vitro* assays. A low intake of carotenoid-rich vegetables decreased lymphocyte proliferation and inhibited the capacity to secrete cytokines, although plasma carotenoid concentrations were not reduced significantly during this period. Supplementing the low-carotenoid diet with vegetable products high in specific carotenoids significantly increased plasma concentrations of these carotenoids. However, only after the tomato juice period were T-lymphocyte functions enhanced compared with the end of the depletion period. This enhancement was paralleled by a significant increase in plasma lycopene concentration (Müller *et al*. 1999).

The reduced lymphocyte proliferative responsiveness observed after 1 week of tomato juice consumption may have been a consequence of the preceeding low-carotenoid period and not an effect of the tomato juice consumption. The different kinetics of the dietary intervention on T-lymphocyte proliferation and IL-2 secretion suggests that IL-2 secretion capacity is more sensitive to such dietary treatments than T-lymphocyte proliferation. Reduction of IL-2 secretion in week 2 preceded the lowered lymphocyte proliferation in week 3 and the recovery in IL-2 secretion in week 3 preceded the recovery in lymphocyte proliferation.

After carrot juice and spinach powder consumption, T-lymphocyte functions were not significantly different compared with week 2 (end of depletion). While at the end of week 6 plasma lycopene concentration had returned to the prestudy concentration, α- and β-carotene and lutein concentrations were increased compared with week 2. These results suggest that tomato juice consumption, as indicated by the high plasma lycopene concentration, is related to the increased cytokine secretion capacity observed after week 4, and tomato juice-specific ingredients may be necessary for adequate T-lymphocyte functions.

Other studies have also investigated the impact of low-carotenoid diets on lymphocyte functions. Consumption of a low-carotenoid diet (β-carotene $< 0{\cdot}1$ mg/d or $= 0{\cdot}5$ mg/d) for 60 d significantly suppressed lymphocyte proliferation without significantly changing plasma β-carotene concentrations (Kramer & Burri, 1997), which helps to confirm the results of the present study. The uptake of a carotenoid complex from vegetables for 20 d corrected the proliferative response (Kramer & Burri, 1997). These results suggest that a β-carotene dosage of 0·5 mg/d may be too low to allow normal proliferation or that the uptake of a mixture of several carotenoids is required in order for lymphocytes to function properly. The results of both studies also question the significance of plasma β-carotene concentrations during β-carotene depletion periods as a factor influencing lymphocyte functions. Probably PBMC carotenoid concentrations more closely mirror the effect of low-carotenoid diets as has been suggested by others recently (Fotouhi *et al*. 1996). In addition, non-carotenoid constituents in carotenoid-rich vegetables could also modulate T-lymphocyte functions. A similar study design was used in another investigation in which β-carotene depletion as well as repletion with pure β-carotene (15 mg/d) did not influence lymphocyte proliferation (Daudu *et al*. 1994). In this study plasma β-carotene concentration significantly decreased during depletion.

In several studies supplementation with β-carotene without using a depletion period before supplementation did not affect lymphocyte proliferation (Ringer *et al*. 1991; Meydani *et al*. 1995*a*). In a study on nutrition and lymphocyte proliferation in young and aged subjects plasma β-carotene concentration also did not correlate with lymphocyte proliferative responsiveness (Gardner *et al*. 1997). Only one study has clearly demonstrated a stimulatory effect on lymphocyte proliferation after supplementation with 30 mg β-carotene/d for 28 d (Moriguchi *et al*. 1996). Besides β-carotene there is no *in vivo* study on lycopene and lymphocyte proliferation and only one study which has investigated the effect of lutein on lymphocyte proliferation. In this animal study a lutein-rich extract from marigold (*Tagetes patula*) was applied orally for 2 and 4 weeks. This treatment resulted in an enhanced lymphocyte proliferation after 2 weeks of supplementation (Chew *et al*. 1996).

IL-2 and IL-4 are cytokines which are both produced by activated T cells. While IL-2 production is concentrated among T-helper-1 cells, IL-4 is primarily produced by T-helper-2 cells (Mosmann & Sad, 1996). IL-2 plays an important role in the proliferation of T cells following *in vitro* stimulation with T-cell mitogens such as ConA. IL-4 has also been shown to stimulate T-cell proliferation. In the present study the low carotenoid intake resulted in a significantly reduced IL-2 secretion capacity. This reduction was paralleled by a suppressed proliferative response of these cells. According to these results carotenoids, or other substances provided by the consumption of carotenoid-rich vegetables, may stimulate proliferation via their impact on IL-2 and/or IL-4 production. No other study looking at the effect of a high carotenoid intake through the consumption of vegetables on cytokine secretion has been published.

Several studies have investigated the effect of pure β-carotene on IL-2 production and on IL-2 receptor expression on activated lymphocytes in human subjects. Supplementation with β-carotene had no effect on IL-2 production in human subjects (Moriguchi *et al*. 1996; Santos *et al*. 1996) or in mice (Chew *et al*. 1996) and did not enhance IL-2 receptor expression on PBMC (Daudu *et al*. 1994; Santos *et al*. 1996). Only in one study using four subjects did β-carotene supplementation result in enhanced IL-2 receptor expression on T lymphocytes (Watson *et al*. 1991). In *in vitro* studies with T-helper-1 and T-helper-2 clones, neither lycopene nor lutein affected the production of a T-helper-1 specific (interferon-γ) and of a T-helper-2 specific cytokine (IL-5) (Iyonouchi *et al*. 1996). Overall the majority of studies clearly show that pure carotenoids do not modulate T-cell cytokine production. This again suggests that probably other constituents in carotenoid-rich vegetables mediate the observed effects on T lymphocytes. In previous studies it has also been shown that β-carotene *in vitro* has no effect on interferon-γ secretion, however, the secretion of monokines (IL-1, tumour necrosis factor α) was significantly increased *in vitro* (Abdel-Fattah *et al*. 1993) and *in vivo* (Hughes *et al*. 1997*a*).

One potential mechanism for an immunomodulatory effect of carotenoids is their ability to act as antioxidants and to quench singlet oxygen, which results in a lower generation of free radicals (Bendich, 1996). Free radicals are known to impair the integrity and functionality of membrane lipids and to affect signal transduction and gene expression in immune cells (Meydani *et al*. 1995*b*). Genotoxicity studies with PBMC from our study subjects revealed that there were fewer oxidized pyrimidine bases of the DNA after carrot juice consumption (Pool-Zobel *et al*. 1997) indicating antioxidative efficacy of carrot juice ingredients. However, the capacity of these PBMC to secrete IL-2 was not significantly different after carrot juice consumption compared with the end of the depletion period, which would not be in line with the proposed hypothesis. Furthermore, no close relationship was observed *in vitro* between the antioxidant activity of carotenoids and their modulating effect on T-helper cell functions (Iyonouchi *et al*. 1996).

The observed differences between the immunomodulatory potential of tomato juice, carrot juice, and spinach powder could be caused at least in part by the different quantities of vegetable products given to the study subjects. While tomato juice and carrot juice at a dose of 330 ml/d provided lycopene 28-fold and β-carotene 10-fold higher than the average intake in Germany, 10 g/d spinach powder provided only five times the quantity of average lutein intake (Pelz *et al*. 1998). In addition, due to the design of our study without the use of wash-out periods, we do not know whether the treatments functioned independently, limiting the interpretation of our results. Because the treatment of the subjects was not randomized we do not know whether the two other vegetable products given after the depletion period would have induced the same results as were achieved with the tomato juice. In future studies the immunomodulatory activity of single vegetable products such as tomato juice should be investigated over a time range allowing the immunological effects to reach stability.

In conclusion, the results of the present study show that in healthy male adults the consumption of a low-carotenoid diet reduced T-lymphocyte functions. Tomato juice consumption restored these functions. During the intervention period the treatment with vegetable products never resulted in the stimulation of T-lymphocyte functions beyond baseline values, although the study subjects showed significantly elevated plasma carotenoid concentrations after the dietary intervention compared with baseline. The active constituents in tomato juice which mediate the immunomodulatory effects, as well as the biological significance of this, must be determined in further investigations.

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Human Nutrition and Metabolism

Moderate Intervention with Carotenoid-Rich Vegetable Products Reduces Lipid Peroxidation in Men[1]

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ABSTRACT Because of their antioxidant properties, carotenoids may have beneficial effects in preventing cancer and cardiovascular disease. However, in humans consuming carotenoid-rich vegetables, data concerning the antioxidant effects of carotenoids are rather scarce. A human intervention trial was conducted, therefore, to determine whether a moderately increased consumption of carotenoid-rich vegetables would influence the antioxidant status in 23 healthy men. This short-term feeding study lasted 8 wk during which the men consumed a low carotenoid diet. A 2-wk low carotenoid period was followed by daily consumption of 330 mL tomato juice, then by 330 mL carrot juice and then by 10 g of spinach powder, each for 2 wk. Antioxidant status [water-soluble antioxidants in serum, ferric reducing ability of plasma (FRAP) and antioxidant enzyme activities] and lipid peroxidation (plasma malondialdehyde and ex vivo oxidation of LDL) were determined. In a subgroup of 10 men, lipoprotein carotenoids were measured. The consumption of carotenoid-rich vegetables significantly increased selected carotenoids in lipoproteins but had only minor effects on their relative distribution pattern. Tomato juice consumption reduced plasma thiobarbituric acid reactive substances (TBARS) by 12% ($P < 0.05$) and lipoprotein oxidizability in terms of an increased lag time (18%, $P < 0.05$). Carrot juice and spinach powder had no effect on lipid peroxidation. Water-soluble antioxidants, FRAP, glutathione peroxidase and reductase activities did not change during any study period. In evaluating the low carotenoid diet, we conclude that the additional consumption of carotenoid-rich vegetable products enhanced lipoprotein carotenoid concentrations, but only tomato juice reduced LDL oxidation in healthy men. J. Nutr. 130: 2200–2206, 2000.

KEY WORDS: • *vegetable* • *humans* • *antioxidant* • *carotenoid* • *lipoprotein*

The oxidative modification of LDL is considered to play an important role in the pathogenesis of atherosclerosis (Diaz et al. 1997). In addition, lipid oxidation products are involved in the formation of mutagenic DNA adducts, which may contribute to carcinogenesis (Chung et al. 1996). It has been hypothesized that dietary antioxidants protect LDL from oxidation and should therefore reduce the risk of atherosclerosis and cancer. In fact, dietary antioxidants such as ascorbic acid, vitamin E and β-carotene have been demonstrated to prevent LDL oxidation in vitro (Frei et al. 1996, Jialal and Devaraj 1996). In vivo studies, however, have yielded contradictory results. Some studies reported that β-carotene supplementation inhibited LDL oxidation (Levy et al. 1996, Nyysönen et al. 1994), whereas others did not find an inhibition of LDL oxidation (Gaziano et al. 1995, Reaven et al. 1993).

Although information exists concerning the absorption, metabolism and action of β-carotene in humans, little is known about these processes for other carotenoids. Nutrition research has focused recently on other carotenoids including α-carotene, lycopene and lutein (Stahl and Sies 1996, Yeum et al. 1996). These include carotenoids to be found in high concentrations in vegetables, such as lycopene in tomato and lutein in spinach, cabbage or kale. One of the major characteristics of these carotenoids is their high antioxidative potential measured as Trolox equivalent antioxidant capacity (TEAC)[4]. Among carotenoids, TEAC values were highest for lycopene, β-carotene and lutein (Miller et al. 1996). Because antioxidant mechanisms are likely involved in the pathogenesis of cardiovascular diseases and cancer (Gaziano and Hennekens 1993, Ziegler 1991), it is tempting to assume that the health benefits associated with the consumption of carotenoid-rich vegetables are due at least in part to antioxidant properties of the carotenoids. This hypothesis is supported by recent studies that showed an increase in plasma antioxidant capacity in humans (Cao et al. 1998a and 1998b, Miller et al. 1998)

[1] Supported by Schoenenberger Pflanzensaftwerke, Magstadt, Germany and Völpel, Königmoos, Germany who kindly supplied the vegetable products.
[2] To whom correspondence should be addressed.
[3] Deceased.

[4] Abbreviations used: CAT, catalase activity; FRAP, ferric reducing ability of plasma; GOR, glutathione reductase; GPX, glutathione peroxidase; GSHt, total glutathione; GSSG, oxidized glutathione; Hb, hemoglobin; PBMC, peripheral blood mononuclear cells; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TEAC, Trolox equivalent antioxidant capacity.

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and protection against lipid peroxidation as measured by thiobarbituric acid reactive substances (TBARS) and breath pentane (Miller et al. 1998) upon increased consumption of fruit and vegetables. Nevertheless, the protection of antioxidant carotenoids from food against LDL oxidation in vivo in humans requires further elucidation (Agarwal and Rao 1998, Rao and Agarwal 1998).

Therefore, we conducted a human intervention trial to determine whether a moderately increased consumption of carotenoid-rich vegetables would elevate plasma carotenoids to a concentration that was correlated negatively with the risk of cancer and cardiovascular disease in epidemiological studies. Effects of carotenoid-rich vegetable products on the antioxidant status and LDL oxidation in men were assessed. Lipoprotein carotenoids were measured to possibly relate diet-induced changes in LDL carotenoids and LDL oxidation measurements.

A study design without washout periods between the different vegetable intervention periods was chosen to mimic more closely the dietary behavior of consumers. Tomato juice, carrot juice and spinach powder were used as sources for specific carotenoids because their major carotenoids showed the highest antioxidant activity in the TEAC assay (Miller et al. 1996). Results of this study referring to plasma carotenoid concentrations (Müller et al. 1999), immunologic effects (Watzl et al. 1999), prevention of lymphocyte DNA damage (Pool-Zobel et al. 1997) and the effect of vegetable products on detoxifying enzymes (Pool-Zobel et al. 1998) have been published.

SUBJECTS AND METHODS

Subjects and study design. The study protocol was described previously (Müller et al. 1999). In brief, 23 nonsmoking men aged 27–40 y in good health, as determined by a screening history and medical check, were examined. Anthropometric data are given in **Table 1**. None were taking vitamin supplements or medication during the study. The study was approved by the Medical Ethical Committee of the Landesaerztekammer Baden-Wuerttemberg and all participants gave their written consent. This short-term feeding study consisted of an 8-wk experimental period, divided into four 2-wk periods. During the study the men consumed a low carotenoid diet. They adhered to their usual diet but were instructed to avoid food products with a high carotenoid content. They were given a list of products to be excluded from the diet (Müller et al. 1999). On the basis of food records, the average daily carotenoid intake from self-selected foods during the study was 1.32 ± 0.58 mg (Watzl et al. 1999). The first 2 wk served as a low carotenoid period, during which no additional carotenoid-rich food was given. For the next 14 d, the subjects ingested 330 mL/d of a commercially available tomato juice (40 mg lycopene) in addition to their meals (tomato period). During wk 5 and 6, the tomato juice was replaced by 330 mL carrot juice (15.7 mg α-carotene and 22.3 mg β-carotene) daily (carrot period). Tomato and carrot juice were provided by Schoenenberger Pflanzensaftwerke, Magstadt, Germany. Finally, during the last 2 wk, a liquid spinach powder preparation (10 g spinach powder; 11.3 mg lutein and 3.1 mg β-carotene; Völpel, Königmoos, Germany) was given with the daily meals (spinach period). **Table 2** describes the carotenoid concentrations of these products.

TABLE 1

Anthropometric data of the men studied[1]

Age, *y*	34 ± 4	(27–40)
Height, *cm*	181 ± 7	(168–200)
Weight, *kg*	76 ± 9	(59–100)
Body fat, *g/100 g*	17 ± 3	(10.7–23.6)
Body mass index, *kg/m*^{2}	23 ± 2	(19.6–28.1)

[1] Values are means ± SD, n = 23 (range).

TABLE 2

Carotenoid concentrations of tomato juice, carrot juice and spinach powder

	Product		
	Tomato juice	Carrot juice	Spinach powder
	mg/100 g		
% Dry matter	6.0	8.7	98
β-Carotene	0.45	6.56	31.1
cis-β-Carotene	0.06	0.19	4.83
α-Carotene	ND[1]	4.76	0.89
β-Cryptoxanthin	ND	0.01	1.47
α-Cryptoxanthin	ND	0.007	ND
Lutein (incl. zeaxanthin)	ND	0.154	113.5
Violaxanthin	ND	ND	22.55
Lycopene	11.84	ND	ND
cis-Lycopene	0.28	ND	ND
Lycopene-oxidation products	0.3	ND	ND
Phytofluene	0.71	0.96	ND
Phytoene	2.23	2.1	ND

[1] ND, not detected.

Blood samples. Blood samples were taken from fasting subjects at the beginning of the study and at the end of each week between 0700 and 0900 h. Blood was drawn from an antecubital vein into prechilled tubes containing EDTA (1.6 g/L, Monovette-Sarstedt, Nümbrecht, Germany) and immediately placed on ice in the dark. Plasma was collected after centrifugation at 1500 × g for 10 min at 4°C. For the lipid peroxidation assay, sucrose (15 g/L) was added to the plasma to prevent LDL aggregation,. For carotenoid analysis, sucrose (15 g/L) and BHT (5 mg/L) as antioxidant were added to the plasma and stored at −80°C until analysis.

Blood antioxidant measurements. Albumin was determined by using a bromcresol green reagent (Boehringer Mannheim, Germany). Bilirubin (DCA method) and uric acid (enzymatic Trinder method) were measured with test kits (RANDOX, Ardmore, N. Ireland). Serum levels of vitamin C were determined spectrophotometrically after derivatization with dinitrophenylhydrazin (Omaye et al. 1979). Intra- and interassay variations were <2 and <6%, respectively.

Glutathione determination. Total glutathione (GSHt) and oxidized glutathione (GSSG) were determined in whole blood, plasma and erythrocytes using a microtiter plate assay based on the method described by Baker et al. (1990) and Richie et al. (1996). Samples were deproteinized using 5-sulfosalicyclic acid (Sigma, Deisenhofen, Germany). The reaction was followed at 405 nm wavelength and 30°C for 30 min at a rate of 1 measurement/min using a temperature-controlled microtiter plate reader (Molecular Devices, München, Germany). GSHt and GSSG concentrations were determined in triplicate and calculated from standard curves of GSH and GSSG (both from Sigma).

FRAP-assay. To measure "antioxidant power," the ferric reducing ability of plasma (FRAP)-assay as described by Benzie and Strain (1996) was used with minor modifications. In brief, in a 96-well microtiter plate, plasma samples (10 μL) were added to 30 μL of H_2O and the reaction was started by further adding 300 μL prewarmed (30°C) FRAP reagent. The reaction mixture was incubated for 8 min at 30°C and absorbance was determined in a microtiter plate reader (MWG Biotech, Ebersberg, Germany) at a wavelength of 585 nm. Intra- and interassay variations were <5%.

Enzyme activity measurements. Superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities in erythrocytes were assayed using commercial test kits (Randox Laboratories, Crumlin, UK), which were adapted to a microplate reader (Molecular De-

vices). Catalase activity (CAT) in erythrocytes was assayed by the method of Aebi (1983). The activity of glutathione reductase (GOR) in plasma was assayed using a slightly modified procedure of Goldberg and Spooner (1983) adapted to a microplate reader. All enzyme assays were done in duplicate or triplicate for individual samples. Intra- and interassay variations were between 5 and 7% and 7 and 9%, respectively. Hemoglobin concentrations in whole blood and erythrocyte hemolysates were analyzed using a Sysmex F-300 analyzer (Sysmex, Hamburg, Germany).

Fatty acid determination. Serum fatty acid components were determined by the method of Müller et al. (1990) on a Fisons 8000 gas chromatograph (Thermoquest, Egelsbach, Germany) using split/splitless injection and flame ionization as detection. The fused silica column (length, 25 m; i.d., 0.25 mm; df, 0.25 μm) coated with chemically bound polyethylene glycol was purchased from Supelco (Sigma). The temperature program started at 50°C (3 min), followed by a rise of 10°C/min to 190°C and a rise of 3°C/min to 230°C (20 min).

Malondialdehyde. Plasma malondialdehyde was determined as thiobarbituric acid reactive substances (TBARS) using a fluorometric method (Yagi 1984). Emission was measured at 548 nm emission wavelength in a fluorescence spectrophotometer (PTI Systems, Wedel, Germany) with an excitation wavelength of 533 nm. Intra- and interassay variations were <4 and <6%, respectively.

Preparation of LDL for oxidation. LDL was isolated by a short-run ultracentrifugation method based on nonequilibrium density-gradient ultracentrifugation (Kleinveld et al. 1992). Centrifugation was carried out in polycarbonate centrifuge tubes by using a Beckman SW-55 Ti rotor at 236,000 × *g* for 2 h at 15°C (Beckman L7–80 ultracentrifuge, Beckman Instruments, Palo Alto, CA). After centrifugation the LDL-containing fraction was located in the upper half of the tube and collected by aspiration. Purity of the LDL fraction was confirmed by agarose gel electrophoresis (Hydragel, Sebia, Fulda, Germany). EDTA and salts were removed from LDL by gel filtration on Pharmacia PD 10 disposable columns (Amersham Pharmacia Biotech, Freiburg, Germany). The LDL oxidation was assayed on the day of preparation.

LDL oxidation. The in vitro oxidation of LDL was performed by using a modification of the procedure described by Esterbauer et al. (1989). The LDL concentration in the PBS solution was determined by measuring total cholesterol with the CHOD-PAP enzymatic test kit (Boehringer) and adjusted for the oxidation assay to 0.1 μmol/L LDL (0.204 mmol/L cholesterol), assuming an LDL molecular weight of 2.5 MDa and a cholesterol concentration of 31.6 g/100 g (Ramos et al. 1995). The LDL oxidation process was followed by recording the conjugated diene absorption at 234 nm in a Perkin Elmer spectrophotometer (Lambda 15, Perkin Elmer, Überlingen, Germany). The instrument was equipped with a water-heated autocell holder for simultaneous measurement of six samples. Oxidation was started by adding $CuCl_2$ to a final concentration of 20 μmol/L. The recording of the 234 nm absorption was started immediately after the addition of $CuCl_2$ and continued at intervals of 3 min for ≤4 h. The recorded absorption data were finally processed on a computer. Intra- and interassay variations were <5 and <8%, respectively.

Isolation and carotenoid analysis of plasma lipoproteins. In a subset of 10 men, carotenoids were analyzed in the major human plasma lipoprotein fractions, i.e., VLDL, LDL and HDL. EDTA plasma with sucrose (15 g/L) and BHT (5 mg/L) added was used for plasma lipoprotein separation by sequential floatation ultracentrifugation adapted to the method of Clevidence and Bieri (1993). Purity of each lipoprotein fraction was determined by agarose gel electrophoresis (Hydragel, Sebia). Ether/ethanol extraction and analysis of carotenoids by HPLC have been described previously (Müller et al. 1999).

Statistics. Results are given as means ± SD, unless otherwise stated. ANOVA and the Friedman test for nonparametric testing were used to compare the depletion period with the different intervention periods. Comparisons of means were performed using the appropriate ANOVA post-test (Tukey-Kramer or Dunn's multiple comparison test). Differences were considered to be significant at $P < 0.05$. Linear regression analysis was performed and the coefficient of correlation (r) was calculated. Statistical calculations were done by using the InStat 2.02 statistical program (Graph Pad Software, San Diego, CA) and StatView 5 (SAS Institute, Cary, NC).

RESULTS

Vegetable juice consumption was well tolerated by all men and none had to be excluded from the study due to illness or noncompliance. Plasma carotenoid concentrations have already been published (Müller et al. 1999).

Carotenoid concentrations in lipoproteins. Lycopene increased significantly in VLDL, LDL and HDL after tomato juice consumption. Both α- and β-carotene were elevated in the lipoprotein fractions after carrot juice consumption and after spinach powder consumption, and lutein was increased in lipoproteins after consumption of a spinach preparation (**Table 3**). After carrot juice consumption, α-tocopherol (4.3 ± 1.3 vs. 6.6 ± 2.2 μmol/L, $P < 0.01$) and ubichinone-10 (39 ± 15 vs.72 ± 33 nmol/L, $P < 0.05$) were significantly reduced in HDL, but not in VLDL and LDL compared with the low carotenoid period.

TABLE 3

Concentrations of carotenoids in plasma lipoproteins of men after consuming a low carotenoid diet and intervention with carotenoid-rich vegetable products[1]

Lipoproteins	Study period	Lutein + Zeaxanthin	α-Carotene	β-Carotene	all-*trans*-Lycopene	*cis*-Lycopene	Lycopene oxides	Phytofluene	Phytoene
					nmol/L				
VLDL	Depletion	28 ± 16	13 ± 9	34 ± 26	11 ± 6	14 ± 9	6 ± 5	15 ± 10	20 ± 12
	Tomato	38 ± 22	16 ± 8	40 ± 21	35 ± 22*	28 ± 17*	10 ± 6	74 ± 44*	103 ± 56*
	Carrot	37 ± 25	98 ± 51*	155 ± 83*	18 ± 13	19 ± 12	10 ± 4	59 ± 40*	86 ± 39*
	Spinach	66 ± 32*	33 ± 14	65 ± 36	18 ± 7	15 ± 6	9 ± 4	33 ± 15	27 ± 14
LDL	Depletion	98 ± 40	127 ± 106	423 ± 350	90 ± 64	118 ± 72	36 ± 16	46 ± 38	48 ± 38
	Tomato	99 ± 40	123 ± 76	424 ± 214	265 ± 76*	245 ± 66*	92 ± 22*	154 ± 62*	221 ± 74*
	Carrot	92 ± 54	828 ± 344*	1230 ± 607*	86 ± 32	108 ± 39	47 ± 14	136 ± 56*	283 ± 114*
	Spinach	215 ± 86*	491 ± 206*	795 ± 396*	89 ± 41	113 ± 37	41 ± 12	89 ± 32*	132 ± 57*
HDL	Depletion	150 ± 55	35 ± 34	91 ± 70	19 ± 8	24 ± 10	9 ± 5	8 ± 6	16 ± 9
	Tomato	132 ± 40	32 ± 26	88 ± 64	39 ± 14*	41 ± 15*	19 ± 8*	13 ± 8	36 ± 20*
	Carrot	114 ± 41	162 ± 60*	194 ± 92*	14 ± 5	15 ± 5	13 ± 4	22 ± 10*	60 ± 33*
	Spinach	281 ± 123*	116 ± 59*	163 ± 101	15 ± 7	19 ± 6	11 ± 2	13 ± 8	31 ± 22

[1] Values are means ± SD, $n = 9$ or 10. * Significantly different from depletion period, $P < 0.05$.

TABLE 4

Relative distribution of carotenoids in plasma lipoproteins of men after consuming a low carotenoid diet and intervention with carotenoid-rich vegetable products[1]

Lipoproteins	Study period	Lutein + Zeaxanthin	α-Carotene	β-Carotene	all-*trans*-Lycopene	*cis*-Lycopene	Lycopene oxides	Phytofluene	Phytoene
		Distribution in lipoprotein fractions, %							
VLDL	Depletion	10.2 ± 6.6	9.2 ± 3.8	7.4 ± 4.8	10.9 ± 5.4	10.1 ± 4.8	12.5 ± 3.1	26.9 ± 12.9	26.7 ± 13.2
	Tomato	14.0 ± 6.9	10.8 ± 5.7	7.7 ± 4.0	10.2 ± 5.4	8.9 ± 4.6	8.3 ± 3.4	29.9 ± 12.8	28.6 ± 11.9
	Carrot	15.3 ± 9.5	9.6 ± 6.0	11.0 ± 7.5	15.2 ± 8.2	13.1 ± 6.8	14.8 ± 7.5	26.5 ± 15.2	20.7 ± 9.6
	Spinach	11.8 ± 4.8	5.3 ± 1.7	6.5 ± 2.4	14.8 ± 2.4	10.3 ± 2.5	14.9 ± 4.8	23.8 ± 8.2	14.8 ± 6.2*
LDL	Depletion	35.6 ± 8.1	70.8 ± 6.9	74.8 ± 5.1	72.1 ± 5.5	74.1 ± 5.2	71.7 ± 7.6	63.4 ± 12.9	54.9 ± 14.2
	Tomato	36.0 ± 5.6	70.5 ± 8.1	77.1 ± 5.1	77.9 ± 6.1	77.8 ± 6.1	75.7 ± 7.2	64.6 ± 12.2	61.6 ± 9.3
	Carrot	36.4 ± 7.1	75.2 ± 6.0	76.6 ± 7.6	72.1 ± 8.3	75.5 ± 7.9	66.7 ± 8.7	63.1 ± 15.4	65.6 ± 12.9
	Spinach	38.7 ± 8.2	76.1 ± 7.5	77.5 ± 6.1	72.4 ± 6.4	75.9 ± 5.4	66.5 ± 6.3	66.9 ± 8.8	68.5 ± 8.4*
HDL	Depletion	54.1 ± 9.8	20.9 ± 6.8	17.8 ± 4.7	17.9 ± 6.4	16.8 ± 7.9	20.2 ± 6.1	13.7 ± 5.7	18.4 ± 4.7
	Tomato	50.0 ± 6.8	18.7 ± 6.4	15.2 ± 4.6	11.9 ± 4.7*	13.3 ± 5.0	16.0 ± 6.4	5.5 ± 2.3*	10.1 ± 4.5*
	Carrot	48.3 ± 7.5	15.2 ± 4.0	12.4 ± 3.6*	12.7 ± 6.0*	11.4 ± 4.2*	18.5 ± 5.2	10.4 ± 4.9	13.7 ± 6.4
	Spinach	49.5 ± 9.2	18.6 ± 7.4	16.0 ± 5.9	12.8 ± 4.9*	13.8 ± 4.9	18.6 ± 3.9	9.3 ± 3.9*	16.7 ± 10.9

[1] Values are means ± SD, $n = 9$ or 10. * Significantly different from depletion period, $P < 0.05$.

Relative distribution of carotenoids in lipoproteins. Consumption of carotenoid-rich vegetable products had only minor effects on the relative distribution of carotenoids among the lipoprotein fractions (VLDL, LDL and HDL; **Table 4**) despite the significant changes in carotenoid concentrations in lipoproteins. In VLDL and LDL, no significant changes in the carotenoid distribution were found except for phytoene, which showed a significant relative reduction in VLDL and an increase in LDL after the spinach period. In HDL, relative reductions of β-carotene, all-*trans*- and *cis*-lycopene, phytofluene and phytoene were observed during various intervention periods (Table 4).

LDL oxidation. Tomato juice consumption for 2 wk reduced lipid peroxidation in healthy men (**Table 5**). The initial concentration of conjugated dienes in LDL did not change during the study. In vitro lipoprotein oxidizability was reduced as seen by an increased lag time (18%) at the end of the tomato juice period ($P < 0.001$). At that point, all-*trans*- and *cis*-lycopene and lycopene oxidation products in plasma and LDL had increased significantly. LDL lycopenes were negatively correlated with lag time (all-*trans*-lycopene, $r = -0.421$, $P = 0.22$; *cis*-lycopene, $r = -0.571$, $P = 0.08$; lycopene oxidation products, $r = -0.816$, $P = 0.004$). There were no significant correlations between lag time and other carotenoids at the end of the tomato juice period. During the carrot and spinach periods, lag time was not elevated compared with baseline values. At the end of the carrot juice period, strong correlations were found between lag time and α- and β-carotene ($r = 0.695$, $P = 0.026$; $r = 0.785$, $P = 0.007$, respectively). No correlation between lag time and lutein was observed after the spinach period. During the tomato juice intervention, plasma TBARS were significantly reduced ($P < 0.05$) by 12% and increased to baseline values at the end of the carrot juice period.

Serum free fatty acids (data not shown) did not change throughout the study.

Water-soluble serum antioxidants. Water-soluble serum antioxidants, uric acid, bilirubin, albumin, glutathione and vitamin C, and the reducing capacity of plasma measured as the FRAP did not change during the vegetable juice intervention (data not shown). Serum uric acid, bilirubin and albumin were within the normal range. Serum vitamin C concentrations ranged from 66.3 ± 12.8 to 73.5 ± 12 μmol/L. There was a strong correlation between uric acid and FRAP ($r = 0.898$, $P < 0.001$) but none for bilirubin, albumin, glutathione or vitamin C.

Compared with the end of the depletion period, tomato juice consumption reduced whole blood GSHt (0.72 ± 0.02 vs. 0.87 ± 0.04 mmol/L, $P < 0.05$) and increased whole blood GSSG (0.09 ± 0.01 vs. 0.07 ± 0.01 mmol/L, $P < 0.05$). Tomato juice had no effect on plasma or erythrocyte GSHt and GSSG. Carrot juice consumption reduced whole blood

TABLE 5

Effect of vegetable juice consumption on LDL oxidation and plasma concentrations of thiobarbituric acid reactive substances (TBARS) in healthy men after consuming a low carotenoid diet and intervention with carotenoid-rich vegetable products[1]

	Study period			
	Depletion	Tomato	Carrot	Spinach
Initial conjugated dienes, *mol/mol cholesterol*	55.9 ± 4.1	57.5 ± 4.2	55.4 ± 3.6	56.4 ± 2.8
Lag time, *min*	106 ± 13.4	124 ± 9.9*	114 ± 12.8	108 ± 11.2
Maximal diene production rate, *mol/mol cholesterol × min*	2.44 ± 0.41	2.15 ± 0.28*	2.11 ± 0.29*	2.25 ± 0.34
TBARS, *μmol/L*	1.62 ± 0.49	1.40 ± 0.36*	1.62 ± 0.51	1.76 ± 0.46

[1] Values are means ± SD, $n = 23$. * Significantly different from depletion period, $P < 0.05$.

GSHt (0.60 ± 0.02 vs. 0.87 ± 0.04 mmol/L, $P < 0.05$) and whole blood GSSG (0.05 ± 0.002 vs. 0.07 ± 0.01 mmol/L, $P < 0.05$) as well as erythrocyte GSHt (0.17 ± 0.01 vs. 0.26 ± 0.01 nmol/10^6 cells, $P < 0.05$), erythocyte GSSG (0.02 ± 0.001 vs. 0.05 ± 0.002 nmol/10^6 cells, $P < 0.05$) and plasma GSSG (1.5 ± 0.1 vs. 2.4 ± 0.3 μmol/L, $P < 0.05$). Plasma GSHt did not change during the carrot juice period. Spinach consumption reduced whole blood GSHt (0.59 ± 0.03 vs. 0.87 ± 0.04 mmol/L, $P < 0.05$) and erythrocyte GSHt (0.13 ± 0.004 vs. 0.26 ± 0.01 nmol/10^6 cells, $P < 0.05$), whereas other glutathione measurements were unaltered. However, these changes did not correlate with any of the carotenoids and derivatives measured in this study.

No significant changes in erythrocyte GPX and plasma GOR activity were observed. Erythrocyte SOD activity [U/g hemoglobin (Hb)] decreased during the low carotenoid period (from 936 ± 206 on d −14 to 816 ± 16 on d 0 ($P < 0.05$) and increased significantly after the first week of tomato consumption (d 7, 961 ± 216 vs. d 0, $P < 0.05$). In contrast, erythrocyte CAT activity (U/g Hb) increased during the low carotenoid period (from 165 ± 21 on d −14 to 184 ± 18 on d 0, $P < 0.05$), was reduced after the tomato period (d 14, 165 ± 24 vs. d 0, $P < 0.05$), but was elevated again after spinach consumption (d 42, 221 ± 34 vs. d 0, $P < 0.05$). All enzyme activities were within the normal ranges (Aebi 1983, Barnett and King 1995, Goldberg and Spooner 1983).

DISCUSSION

It was our aim to evaluate whether a moderate short-term dietary intervention with carotenoid-rich vegetable products affects antioxidant status and LDL oxidation in humans. We further wanted to investigate whether diet-related increases in lipoprotein carotenoids might be responsible for changes in lipoprotein oxidation.

A study design without washout periods between the different vegetable intervention periods was chosen to mimic more closely the dietary behavior of consumers. Although we measured carotenoid concentrations in plasma and lipoproteins, we cannot exclude relevant functional carryover effects of individual carotenoids at the end of each study period. In a comparable study without washout periods between the interventions, the effect of tomato consumption on lymphocyte DNA damage was investigated (Riso et al. 1999). Although a significant carryover effect was observed with lycopene plasma concentration, no carryover effect was present when lymphocyte resistance to oxidative stress was measured. This suggests that functional carryover effects may not be the major limitation with this study design. During the intervention, the men had a different carotenoid uptake with the vegetable products. They ingested 330 mL tomato juice (40 mg lycopene), 330 mL carrot juice (15.7 mg α-carotene and 22.3 mg β-carotene) and 10 g spinach powder (11.3 mg lutein and 3.1 mg β-carotene) daily. It was our objective to study the health effects of reasonable serving sizes of vegetable products and not to compare different carotenoids on an equimolar basis. As a result of this approach, we cannot exclude the possibility that the different doses of carotenoids may have influenced the antioxidant measurements.

In this study, the consumption of carotenoid-rich food increased lipoprotein carotenoids, showing that the major carotenoid in the food is also the major carotenoid appearing in lipoproteins. The intake of carotenoid-rich vegetable products had no effect on relative distribution of carotenoids in VLDL and LDL, except for phytoene. This might be caused by the ingestion of the phytoene-free spinach preparation after 4 wk consumption of phytoene-rich products, which can be seen by the overall decrease of the phytoene concentration in all lipoprotein fractions. Similarly, the relative changes in HDL carotenoid distributions may be explained by the corresponding changes in HDL carotenoid concentrations. The relative distribution pattern of carotenoids among lipoprotein fractions in this study is consistent with previous reports (Clevidence and Bieri 1993, Paetau et al. 1998). Taken together, the data show that the appearance of carotenoids in lipoproteins reflects the uptake from carotenoid-rich food and does not substantially influence the relative distribution pattern, although plasma carotenoid concentrations increased several fold.

Tomato juice consumption for 2 wk significantly increased plasma and lipoprotein lycopene concentrations. Compared with the other two vegetable products, tomato juice was most effective in this study as observed by the reduced lipid peroxidation product TBARS in plasma and the ex vivo oxidation of LDL, with an increased lag time and reduced diene production rates. This suggests that lycopene accounts for the LDL-protecting effect during the tomato juice intervention period because plasma and LDL lycopene increased significantly after tomato juice consumption. However, other compounds from tomato juice may also protect LDL from oxidation, contributing to the prolonged lag time. Nevertheless, in vitro studies have shown that lycopene is a potent antioxidant (DiMascio et al.1989, Miller et al. 1996, Woodall et al. 1997) that protects LDL (Oshima et al. 1996, Romanchik et al.1997) and other lipid structures from oxidation (Klebanov et al.1998, Stahl et al. 1998). To date, there is little information on the effects of tomato consumption on markers of oxidative stress in vivo. Recently, Sutherland et al. (1999) found that tomato juice consumption (400 mL/d, 4 wk) increased plasma lycopene concentrations but had no effect on lipid peroxidation (lag time, TBARS, LDL lipid peroxides) in 15 cyclosporine-treated patients with stable kidney transplants. In that study, baseline plasma lycopene concentrations in the patients were approximately five times higher than in healthy men. Cyclosporine treatment, which may increase susceptibility of LDL to oxidation (Apanay et al. 1994), and the high baseline plasma lycopene concentrations may account for the lack of observed effect in the kidney transplant patients. On the other hand, Agarwal and Rao (1998) and Rao and Agarwal (1998) showed that consumption of tomato products reduced lipid peroxidation and DNA damage in humans as seen in the reduction of serum and LDL TBARS and lymphocyte 8-oxo-2′-deoxyguanosine content. In a previous paper from our intervention trial, we also found reduced DNA damage, measured as single strand breaks in peripheral blood mononuclear cells (PBMC), after tomato juice consumption (Pool-Zobel et al. 1997). Whether these protective effects of tomato juice are mediated by lycopene alone or by other antioxidants such as polyphenols and phenolic acids present in tomatoes, could not be determined here because we did not measure other tomato-specific phytochemicals in plasma. However, we determined the major antioxidants in plasma and lipoproteins. Concentrations of vitamin C, α-tocopherol and ubichinone-10 in plasma, and α-tocopherol and ubichinone-10 in LDL were not affected by any of the dietary interventions in this study and may therefore not contribute to the observed antioxidant effects after tomato juice consumption. The serum fatty acid composition, which also influences the oxidizability of LDL (Thomas et al.1994, Tsimikas and Reaven 1998) did not change during the study. We conclude, therefore, that the serum fatty acid composition does not contribute to the reduced LDL oxidizability after tomato juice consumption.

In contrast to tomato juice, carrot juice had less pronounced effects on antioxidant defense. No changes in plasma TBARS and lag time were found. However, the diene production rate in the ex vivo LDL oxidation assay was reduced significantly after 2 wk of carrot juice consumption. We also found a decrease in oxidative DNA damage in PBMC at this time point (Pool-Zobel et al.1997). To date, studies on the antioxidant properties of carotenoids in humans have been designed using β-carotene as a supplement, which reduced lipoprotein oxidation in healthy volunteers (Levy et al. 1996, Nyysönen et al.1994), children suffering from cystic fibrosis (Winklhofer-Roob et al.1995) and coronary artery patients (Mosca et al.1997). However, some studies showed that supplementation with β-carotene in vivo did not inhibit LDL oxidation (Gaziano et al. 1995, Reaven et al. 1993). In these studies, rather high doses (50–100 mg) of β-carotene were given, and the authors discussed their results as possible prooxidant effects of increased β-carotene levels at least during the in vitro assay of LDL oxidation. These findings and the results of the ATBC (Hennekens et al. 1996) and CARET studies (Omenn et al.1996), in which β-carotene supplementation even increased lung cancer risk, suggest that the consumption of whole diets rich in carotenoids (and other phytochemicals) and not the supplementation with single compounds may be important to prevent LDL oxidation and/or disease development.

The findings of Hininger et al. (1997) support this conclusion. They showed an inhibition of the susceptibility of LDL to oxidation after carotenoid-rich food intake for 2 wk in which carrots, tomatoes, and cabbage + spinach provided an additional daily amount of 10 mg β-carotene, 10 mg lycopene and 10 mg lutein. Compared with our intervention trial, which increased plasma carotenoids several fold, those authors reported only minor effects on plasma carotenoid concentrations. In the nonsmoking group, which is comparable to our study group, only α- and β-carotene and retinol increased significantly in plasma. They concluded that the protective effect of fruit and vegetables on susceptibility of LDL to oxidation may also be related to biological interactions between carotenoids and other antioxidants and possible synergistic effects. The study of Hininger et al. (1997) and our present and earlier (Pool-Zobel et al. 1997) results are indicative of an increased antioxidant capacity in blood lipid fractions and cells as a result of the consumption of vegetable juice. This increase in antioxidant capacity could be explained by the increase in blood carotenoid levels. However, to be able to make such a conclusion, we had to study the effects of the vegetable consumption on endogenous antioxidants and systems involved in the detoxification of reactive oxygen species.

GSH is one of the endogenous antioxidants that plays an important role in the cellular defense against reactive oxygen species. The GSHt and GSSG concentrations measured in this study fit very well with concentrations reported for blood, plasma and erythrocytes of healthy volunteers (Costagliola et al. 1990, Henning et al. 1991, Hininger et al. 1997). Interestingly, GSHt and GSSG levels in blood, plasma and erythrocytes were decreased significantly at different sampling periods throughout the study compared with GSHt and GSSG concentrations at the end of the carotenoid depletion phase (d 0). However, due to the diversity and the inconsistency of the decreases in GSHt and GSSG concentrations, the relationship with the vegetable intervention remains unclear. The decrease in GSHt concentrations in blood and erythrocytes during the intervention with carrot juice and spinach powder was ~25%, which is on the same order of magnitude as the difference in GSHt concentration observed between smokers and nonsmokers (Costagliola et al. 1990, Hininger et al. 1997). It is therefore tempting to speculate about a possible relationship between cellular GSH and the consumption of vegetables, an area of investigation requiring further research.

Other water-soluble antioxidants in serum (albumin, bilirubin, uric acid, ascorbic acid), the antioxidant power (FRAP) and the enzymes GPX and GOR did not change during the study. For the spinach period, our results are comparable to those of Castenmiller et al. (1999), who studied the effect of carotenoid supplementation and spinach intake on blood antioxidant enzyme activities and FRAP. One group ingested 11.5 mg of lutein daily from spinach products for 3 wk. In that study, consumption of spinach had no effect on FRAP, GPX, SOD, GOR and CAT when comparing wk 0 with wk 3. This is in agreement with our findings except for CAT, for which we found an increase in activity after spinach consumption. Looking at another tomato juice intervention, Böhm and Bitsch (1999) recently showed that tomato juice consumption (5 mg/d lycopene, 2 wk) significantly increased plasma lycopene concentrations. However, total plasma antioxidant activity was not altered significantly by tomato juice intervention. These results and our findings suggest that lipid-soluble carotenoids from vegetable products do not substantially influence water-soluble antioxidants, antioxidant power and antioxidant enzyme activities in healthy humans.

In conclusion, our data show that the appearance of carotenoids in lipoproteins reflects the uptake from carotenoid-rich food and does not substantially influence the relative carotenoid distribution pattern in lipoproteins, although plasma carotenoid concentrations increased several fold. Tomato juice, but not carrot juice or spinach powder consumption reduced LDL oxidation in healthy men. Our findings also suggest that the consumption of carotenoid-rich vegetables does not influence water-soluble antioxidants and antioxidant power and may have only minor effects on antioxidant enzyme activities in healthy men.

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Human Nutrition and Metabolism

Prolonged Tomato Juice Consumption Has No Effect on Cell-Mediated Immunity of Well-Nourished Elderly Men and Women[1]

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ABSTRACT The immunomodulatory potential of carotenoids has been investigated thoroughly only for β-carotene. Data on the immunomodulatory activity of other carotenoids such as lycopene are scarce. The objective of this study was to investigate the effects of prolonged tomato juice consumption on cell-mediated immunity of well-nourished healthy elderly persons. In an intervention study, 33 female and 20 male subjects (aged 63–86 y) consumed 330 mL/d tomato juice (47.1 mg/d lycopene) or mineral water for 8 wk. Immune status was assessed by measuring number and lytic activity of natural killer (NK) cells, secretion of cytokines [interleukin (IL)-2, IL-4, tumor necrosis factor-α (TNF-α)] by activated peripheral blood mononuclear cells (PBMC), lymphocyte proliferation, and delayed-type hypersensitivity (DTH) skin responses. Tomato juice consumption resulted in significantly increased plasma lycopene and β-carotene concentrations over time. In both treatment groups, TNF-α and IL-4 secretion were increased at the end of the intervention period, whereas IL-2 secretion was decreased. Tomato juice consumption had no effect on lymphocyte proliferation, DTH or the number of NK cells. Lytic activity of NK cells was increased in both groups at the end of the intervention period. In conclusion, these results show that prolonged tomato juice consumption increased plasma lycopene concentrations without significantly affecting cell-mediated immunity in well-nourished elderly subjects. J. Nutr. 130: 1719–1723, 2000.

KEY WORDS: • *tomato juice* • *lycopene* • *elderly humans* • *cell-mediated immunity*

A high consumption of tomato and tomato-based products is associated consistently with a low risk of cancer for a variety of anatomic sites (Giovannucci 1999). Numerous potentially beneficial compounds are present in tomatoes, with lycopene as the major phytochemical (Beecher 1998). Although the active compounds in tomatoes contributing to the observed reduction of cancer risks are presently unknown, the benefits of tomatoes are often attributed to lycopene (Clinton 1998), and results from a recent study support this assumption (Gann et al. 1999). Few studies to date have looked at the immunomodulatory activity of lycopene, which may contribute to the observed cancer risk reduction with high consumption of tomato products. In vitro, lycopene suppressed T-helper 2 (TH2)[3] cell clone–mediated antibody production in unprimed spleen cells. It had no effect on interleukin (IL)-5 production of TH2 cells (Iyonouchi et al. 1996). In a mouse model, during tumorigenesis, lycopene normalized the change of intrathymic T-cell differentiation by increasing the CD4+ cells (Kobayashi et al. 1996). In a human intervention trial, supplementation with 15 mg/d lycopene for 28 d induced no changes in monocyte surface marker expression of healthy adults (Hughes et al. 1997).

We have recently shown that in healthy adults a low carotenoid diet resulted in significantly reduced proliferation of peripheral blood mononuclear cells (PBMC). In addition, secretion of IL-2 and IL-4 by these cells was also significantly decreased. After 2 wk of supplementation with 330 mL/d tomato juice, lymphocyte functions were normalized (Watzl et al. 1999), whereas plasma lycopene concentrations were significantly higher compared with baseline (Müller et al. 1999). DNA from PBMC of these subjects had significantly lower endogenous levels of strand breaks, indicating that tomato juice consumption induced protective mechanisms in PBMC (Pool-Zobel et al. 1997). These results have been confirmed by a recent study, which reported increased resistance of lymphocyte DNA to oxidative damage after tomato consumption (Riso et al. 1999). Whether enhanced antioxidative protection of PBMC after tomato consumption is related to an immunomodulatory activity of tomato compounds such as lycopene is currently not known.

The results of our first study prompted us to conduct an intervention trial with healthy, noninstitutionalized elderly people instead of young subjects to investigate the effect of prolonged tomato juice consumption on cell-mediated immunity. Elderly subjects have a dysregulation of immune re-

[1] Presented in poster form at Experimental Biology 99, April 20, 1999, Washington, DC [Watzl, B., Bub, A., Blockhaus, M., Müller, H., Herbert, B., Lührmann, P., Neuhäuser-Berthold, M. & Rechkemmer, G. (1999) Prolonged tomato juice consumption has no effect on immune function and on plasma antioxidant activity of well-nourished elderly. FASEB J. 13: A590 (abs.)].

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[3] Abbreviations used: ConA, concanavalin A; DTH, delayed-type hypersensitivity; FBS, fetal bovine serum; IL, interleukin; NK, natural killer; PBMC, peripheral blood mononuclear cells; TH, T helper; TNF-α, tumor necrosis factor-α.

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sponses, mainly as a result of changes in cell-mediated immunity (Lesourd 1997). Therefore, any immunostimulatory activity of tomato or tomato-specific phytochemicals such as lycopene should be seen more clearly in subjects with age-related impaired immune functions.

SUBJECTS AND METHODS

Subjects. Subjects were participants of the Longitudinal Study in an Aging Population of Giessen (GISELA), Germany, in which the nutritional and health status of free-living elderly people is investigated at yearly intervals. Of the 453 subjects included in the survey, selection for the participants of the tomato study was based on the following exclusion criteria: smoking, alcohol consumption > 50 g/d, food allergies, cancer, coronary heart disease, chronic inflammatory diseases (rheumatoid arthritis, Crohn's disease, colitis ulcerosa), diabetes, asthma, use of prescription medication or nonsteroidal anti-inflammatory drugs on a regular basis, use of vitamin or mineral supplements for the last 3 mo, corticosteroid treatment and intake of immunostimulators for the last 4 wk. Fifty-three elderly subjects were enrolled after screening. The study was approved by the Ethical Committee of the Department of Medicine, Justus-Liebig-University Giessen and all participants gave their consent in writing.

Study design. This study was conducted during the months of May–July, 1998. Subjects were assigned randomly to the control or intervention group. Energy and nutrient intakes of the study subjects before the study period were assessed by means of a validated 3-d estimated food record (Lührmann et al. 1999). Subjects of the tomato juice group consumed daily 330 mL tomato juice (47.1 mg lycopene, 1.7 mg β-carotene; Schoenenberger, Magstadt, Germany) for 8 wk. Subjects of the control group consumed the same volume of mineral water. Subjects were instructed to drink tomato juice or water with their main meal. Because the current intake of lycopene in Germany is ~1 mg/d (Pelz et al. 1998) and the tomato juice provided ~40 times more lycopene, subjects were allowed to continue with their regular diet throughout the study period including tomato products in the control group. During the entire intervention period, subjects were asked to protocol any diseases or medicine use. Blood samples from fasting subjects were collected between 0700 and 1030 h.

Measurement of carotenoids and ascorbic acid. Carotenoids in tomato juice, fetal bovine serum (FBS) and plasma were measured by reversed-phase HPLC as described earlier (Müller et al. 1999). Plasma ascorbic acid concentrations were determined by a modified 2,4-dinitrophenylhydrazine method (Lowry et al. 1943).

Isolation of PBMC and preparation of serum. Blood was drawn into K^+-EDTA tubes (proliferation and cytokine secretion) and lithium heparin tubes [natural killer (NK) cell lytic activity]. PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma, Deisenhofen, Germany) and resuspended in complete RPMI-1640 culture medium (Life Sciences, Eggenstein-Leopoldshafen, Germany), containing 5% (v/v) heat-inactivated FBS (Life Sciences), L-glutamine (2 mmol/L), penicillin (100,000 U/L) and streptomycin (100 mg/L). Serum from each subject was heat inactivated for 30 min at 56°C.

Lymphocyte proliferation. PBMC at 1×10^9 cells/L in medium containing 5% of either FBS or autologous serum were stimulated by the T-cell mitogen concanavalin A (5 mg/L, ConA, Sigma) for 120 h at 37°C. Proliferation was measured using the thymidine analog 5-bromo-deoxyuridine, which was quantitated in PBMC by a cellular enzyme immunoassay as described earlier (Watzl et al. 1999).

Quantification of cytokine secretion. PBMC at 1 x 10^9 cells/L were cultured in medium containing 5% of either FBS or autologous serum and stimulated by 5 mg/L ConA for 48 h at 37°C (IL-2, IL-4) or by 10 mg/L lipopolysaccharide (Difco, Augsburg, Germany) for 24 h at 37°C (TNF-α). Cell-free supernatants were collected and stored at −80°C until analysis. IL-2 and IL-4 were measured by sandwich-ELISA as described earlier (Watzl et al. 1999). For TNF-α, a sandwich-ELISA was developed using an anti-human TNF-α monoclonal antibody (25 mg/L PBS, pH 7.4; Endogen, Eching, Germany) as capture antibody and a monoclonal biotin-labeled mouse anti-human TNF-α antibody [375 μg/L PBS-Tween and 4% v/v bovine serum albumin (BSA); Endogen] as detection antibody.

Percentage and lytic activity of NK cells. The percentage of NK cells was determined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany). PBMC were incubated with a phycoerythrin-conjugated monoclonal antibody NCAM16.2/anti-CD56 (Becton Dickinson) and a FITC-conjugated monoclonal antibody anti-CD3 (Becton Dickinson), washed and fixed with 1% paraformaldehyde (Sigma). Lytic activity of NK cells against K562 target cells (effector: target ratios 50:1, 25:1, 12.5:1) was measured with a recently described flow cytometric method (Chang et al. 1993) and calculated as the percentage of dead target cells in the test samples minus the percentage of dead target cells in the control samples without effector cells.

Assessment of delayed-type hypersensitivity (DTH). DTH skin response was assessed with Multitest-CMI (GN Pharma, Fellbach, Germany) loaded with glycerine control and seven common antigens. The device was administered on the forearm by a person who was blinded to the study treatment assignment. The diameter of positive reactions was measured 48 h after administration of the test. According to the manufacturer's instruction, an induration of ≥2 mm was considered positive.

Statistical analyses. Normal distribution of the data was analyzed by using the Kolmogorov-Smirnov normality test. Baseline data vs. post-treatment data within groups were analyzed by using Student's paired *t* test or Wilcoxon's rank test for data that are not normally distributed. Differences between treatment groups were analyzed by using Student's *t* test for independent samples (or the Mann-Whitney U test for data that were not normally distributed) on mean pre- to postintervention differences. To assess the plasma lycopene-NK lytic activity relationship, Spearman correlation coefficients were computed. Statistical significance was accepted at the $P < 0.05$ level. All statistical calculations were performed with the StatView program (SAS Institute, Cary, NC).

RESULTS

Three subjects were excluded from the study because they had to receive a drug treatment during the intervention period; 50 subjects completed the study with 21 (13 women, 8 men) subjects in the control and 29 (19 women, 10 men) in the tomato juice group. No other subjects reported any intercurrent diseases during the study. On the basis of the results of the 3-d estimated food record, there were no differences between groups at the beginning of the study in the intake of energy, protein, carbohydrates and fat as well as in vitamin E and zinc (data not shown). The β-carotene and ascorbic acid intakes before the study period also did not differ between groups (control 4.57 ± 2.59 mg/d and 116.5 ± 49.9 mg/d, tomato juice group 4.24 ± 2.53 mg/d and 119.1 ± 61.5 mg/d). Plasma ascorbic acid concentrations were measured only at the end of the study period and did not differ between groups (control 72.7 ± 18.2 μmol/L, tomato juice group 72.7 ± 14.2 μmol/L). No differences were observed between groups in average age (control 70.5 ± 5.2 y, tomato juice 69.7 ± 5.8 y) and body mass index (control 26.7 ± 3.0 kg/m^2, tomato juice 26.6 ± 2.4 kg/m^2). All subjects maintained body weight throughout the study (data not shown).

There was no significant difference in plasma carotenoid concentrations between groups at baseline (**Table 1**). Tomato juice consumption significantly increased plasma concentrations of all-*trans*- and *cis*-lycopene (threefold), α-cryptoxanthin and β-carotene. (Table 1). No carotenoids or tocopherols were detected in FBS.

The capacity of PBMC (cultured in medium and FBS) from both groups to secrete the cytokines TNF-α, IL-2 and IL-4 did not differ significantly at baseline (**Table 2**). During the intervention, the capacity of PBMC to secrete TNF-α increased significantly in the tomato group and tended to increase in the control group ($P = 0.058$). A significantly higher production of IL-4 was also seen in both groups. In contrast, IL-2 secretion decreased in both groups after 8 wk of intervention. Similar

TABLE 1

Plasma carotenoid concentrations of elderly subjects before and after consuming mineral water or tomato juice for 8 wk[1]

	Week 0		Week 8	
	Control	Tomato	Control	Tomato
			μmol/L	
Lutein	0.38 ± 0.22	0.32 ± 0.19	0.39 ± 0.19	0.32 ± 0.18
α-Cryptoxanthin	0.036 ± 0.02	0.046 ± 0.04	0.036 ± 0.02	0.062 ± 0.04*
β-Cryptoxanthin	0.16 ± 0.07	0.18 ± 0.14	0.14 ± 0.05	0.16 ± 0.12
all-*trans*-Lycopene	0.16 ± 0.10	0.13 ± 0.07	0.18 ± 0.12	0.54 ± 0.21**
cis-Lycopene	0.11 ± 0.07	0.13 ± 0.19	0.12 ± 0.08	0.46 ± 0.17**
α-Carotene	0.22 ± 0.29	0.19 ± 0.14	0.23 ± 0.31	0.21 ± 0.09
all-*trans*-β-Carotene	0.68 ± 0.58	0.75 ± 0.42	0.79 ± 0.56	1.10 ± 0.48**
cis-β-Carotene	0.042 ± 0.03	0.047 ± 0.02	0.052 ± 0.03	0.077 ± 0.04**

[1] Values are means ± SEM, $n = 21$ (control) or 29 (tomato). * $P < 0.01$, ** $P < 0.001$ wk 0 vs. wk 8 and control vs. tomato.

effects were seen with PBMC cultured in medium and autologous serum (Table 2).

Lymphocyte proliferation did not differ between groups at baseline or after 8 wk of mineral water and tomato juice consumption (Table 2). ConA-activated lymphocytes were also cultured in medium and autologous serum, and no differences were observed between groups at either time point.

Flow cytometry revealed no significant differences in the percentages of NK cells between groups at baseline and after the dietary intervention (**Table 3**). The lytic activity at baseline did not differ between the groups, whereas during the intervention period, lytic activity increased significantly in both groups. A significant correlation between plasma all-*trans*-lycopene concentrations and lytic activity of NK cells ($r = 0.367$, $P = 0.049$) was seen in the tomato juice group. Because we observed an increase in both groups at all effector:target ratios, only the results for the 25:1 ratio are shown in Table 3.

DTH skin response was measured as the total number of positive skin reactions (antigen score) and as the total diameter of induration of all positive reactions (cumulative score). There were no significant differences in antigen score and cumulative score at baseline or after 8 wk of intervention. However, in both groups, antigen score and cumulative score increased during the intervention period (Table 3).

TABLE 2

Cytokine production and proliferation of peripheral blood mononuclear cells [cultured in medium containing 5% fetal bovine serum (FBS) or autologous serum (AS)] from elderly subjects before and after consuming mineral water or tomato juice for 8 wk[1]

	Week 0		Week 8	
	Control	Tomato	Control	Tomato
		Tumor necrosis factor-α, μg/L		
FBS	11.4 ± 1.2	11.0 ± 0.8	13.4 ± 1.2	13.9 ± 0.8**
AS	9.9 ± 1.3	9.7 ± 1.0	12.3 ± 1.5	11.2 ± 0.8
		Interleukin-2, ng/L		
FBS	458 ± 77	406 ± 73	273 ± 82*	269 ± 65**
AS	202 ± 42	140 ± 32	140 ± 36*	116 ± 26
		Interleukin-4, ng/L		
FBS	20.9 ± 3.1	18.4 ± 5.6	32.1 ± 4.0**	35.6 ± 5.5**
AS	5.3 ± 1.6	6.9 ± 2.4	11.1 ± 1.7**	13.8 ± 3.6***
		Lymphocyte proliferation (A_{450}-A_{650})		
FBS	1.31 ± 0.11	1.32 ± 0.11	1.35 ± 0.11	1.24 ± 0.09
AS	2.11 ± 0.13	1.77 ± 0.14	1.92 ± 0.13	1.75 ± 0.12

[1] Values are means ± SEM, $n = 21$ (control) or 29 (tomato). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ wk 0 vs. wk 8.

DISCUSSION

The immunomodulatory potential of carotenoids has been investigated thoroughly only for β-carotene. Data on the immunomodulatory activity of other carotenoids such as lycopene are scarce. We showed recently that 2 wk of tomato juice consumption (supplying 40 mg/d lycopene) after a 2-wk period of a low carotenoid diet stimulated lymphocyte proliferation and IL-2 secretion of PBMC in healthy adults (Watzl et al.

TABLE 3

Percentage of natural killer (NK) cells, NK cell activity of peripheral blood mononuclear cells and delayed-type hypersensitivity skin response of elderly subjects before and after consuming mineral water or tomato juice for 8 wk[1]

	Week 0		Week 8	
	Control	Tomato	Control	Tomato
		NK cells		
%	18.5 ± 2.5	18.2 ± 1.8	19.9 ± 2.7	18.0 ± 1.8
Activity[2]	39.7 ± 4.5	35.1 ± 3.0	55.7 ± 4.2*	45.6 ± 3.3*
		Delayed-type hypersensitivity		
A[3]	2.3 ± 0.3	2.1 ± 0.2	2.7 ± 0.3	2.7 ± 0.2
B[4]	10.0 ± 1.3	9.5 ± 1.3	12.5 ± 1.5*	11.2 ± 1.1

[1] Values are means ± SEM, $n = 21$ (control) or 29 (tomato). [2] % lysed target cells. [3] (A) Antigen score calculated as the total number of positive reactions. [4] (B) Cumulative score calculated as the total diameter of induration of all positive reactions in mm. * $P < 0.001$ wk 0 vs. wk 8.

1999). The objective of this study was to investigate the effects of prolonged tomato juice consumption on cell-mediated immunity of well-nourished healthy subjects with a higher risk for impaired immune functions such as elderly people.

Tomato juice consumption significantly increased plasma lycopene and β-carotene concentrations. In relation to the average plasma β-carotene and ascorbic acid concentrations of elderly subjects (age > 65 y) in Germany (Heseker et al. 1992) and other countries (Pallast et al. 1999, Vogel et al. 1997), our study subjects showed higher plasma β-carotene and ascorbic acid concentrations. This suggests that the participants in our study have been well-nourished, which is of relevance for the interpretation of the immunological results.

In our study, we sought to determine whether tomato juice consumption affects cytokine secretion capacity of PBMC from elderly subjects. The majority of studies in elderly subjects show that lymphocytes produce significantly less TH1 subset–derived cytokines such as IL-2 and more TH2 cytokines such as IL-4, indicating a dysregulation between TH1 and TH2 subsets (Rink et al. 1998). The production of proinflammatory cytokines such as TNF-α seems to be enhanced in elderly people compared with young adults (Rink et al. 1998). During our intervention, the secretion capacity of PBMC for TNF-α and IL-4 was increased in both groups, whereas subjects in both groups showed reduced IL-2 production after 8 wk of tomato juice consumption. These results clearly indicate that tomato juice consumption (i.e., a high intake of lycopene) does not interfere with the regulation of cytokine secretion capacity in elderly subjects. This conclusion is supported by our findings that PBMC cultured in medium containing 5% FBS (free of carotenoids and tocopherols) or 5% autologous serum (high concentrations of tomato-derived lycopene) yielded similar results. The reason for the significant changes in cytokine secretion observed in both groups is unknown.

Reduced lymphocyte proliferation is a common phenomenon observed in elderly subjects (Lesourd 1997), and enhancement of this T-cell function by dietary means would be an interesting approach to stimulate immune functions in this vulnerable population. In our study, proliferation of activated PBMC cultured with medium containing 5% FBS or autologous serum was not affected by the dietary intervention and did not differ between groups. This suggests that tomato juice–derived compounds in PBMC or in autologous serum do not influence lymphocyte proliferation in vitro. However, the addition of tomato juice to a low carotenoid diet stimulated lymphocyte proliferation in adults (Watzl et al. 1999). No other studies have looked at the effect of tomato juice and/or lycopene on lymphocyte proliferation.

Alterations of the immune system during aging include functions of NK cells (Solana et al. 1999). The results of our study show that the percentage of NK cells in PBMC was not affected by the dietary intervention. In addition, lytic activity of NK cells was increased independently of the dietary treatment. However, a weak significant positive correlation between plasma all-*trans*-lycopene concentrations and lytic activity of NK cells was seen in the tomato juice group. This suggests that high plasma all-*trans*-lycopene concentrations may stimulate NK cell activity. Because we did not observe differences between treatment groups and multiple factors modulate this activity, we cannot speculate at this time about potential mechanisms of lycopene.

The age-related impaired cell-mediated immunity is reflected in the inability of elderly people to mount a DTH (Bogden and Louria 1997), and a low DTH response has been shown to be associated with increased mortality in healthy elderly people (Wayne et al. 1990). In both groups of our study, the range for the antigen score and cumulative score was comparable to those observed in other studies of healthy elderly subjects (Girodon et al. 1999, Pallast et al. 1999, Santos et al. 1997), suggesting a normal DTH response in our elderly subjects. DTH was not significantly affected by the dietary intervention in our subjects. There are no other studies in the literature investigating the effect of carotenoid-rich vegetables or lycopene on DTH.

Studies have shown that tomato juice consumption reduces oxidative DNA damage in PBMC (Rao and Agarwal 1998, Riso et al. 1999). Prevention of such oxidative damage is one potential mechanism for an immunomodulatory effect of carotenoids (Meydani et al. 1995). Although we found a weak positive correlation between plasma all-*trans*-lycopene and NK cell activity, no effect of tomato juice consumption on cell-mediated immunity was observed.

In conclusion, our study suggests that in well-nourished elderly people, supplementation with tomato juice resulting in high plasma lycopene levels does not modulate cell-mediated immunity. It is likely that the good nutritional status of our elderly subjects led to their normal immune functions. This hypothesis is supported by the results of our study with young healthy adults consuming a low carotenoid diet. Supplementation with tomato juice significantly stimulated various T-lymphocyte functions in these subjects (Watzl et al. 1999).

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Paraoxonase 1 Q192R (PON1–192) polymorphism is associated with reduced lipid peroxidation in R-allele-carrier but not in QQ homozygous elderly subjects on a tomato-rich diet

Summary *Background* The oxidative modification of LDL is considered to play a central role in the pathogenesis of atherosclerosis and coronary heart disease (CHD). Paraoxonase (PON1) protects LDL from oxidation and may therefore retard the developement of atherosclerosis. The PON1–192 polymorphism is associated with diminished PON1 concentrations and an increased risk for CHD in RR-allele subjects. *Aim of the study* To investigate the effect of tomato juice consumption on PON1 activity and other parameters related to oxidative stress in healthy elderly subjects. Furthermore, the PON1–192 genotype has been determined in the volunteers in order to see whether possible treatment effects are related to the PON1–192 polymorphism. *Methods* Fifty elderly subjects were randomly assigned to control (mineral water) or intervention group (tomato juice). Subjects of the tomato juice group consumed daily 330 mL tomato juice for 8 weeks. Antioxidant status was measured as LDL oxidation, plasma malondialdehyde, ferric reducing ability of plasma (FRAP) and PON1 activity. The PON1–192 polymorphism was determined by restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR). Plasma carotenoids were analyzed by HPLC. *Results* Tomato juice consumption reduced LDL-oxidation and improved antioxidant status in R-allele carriers, but not in the QQ genotype group. PON1 activity increased irrespective of the genotype in both, control and intervention group. *Conclusions* The changes in antioxidant status after tomato juice consumption seem to depend on the PON1–192 genotype. Healthy elderly, carrying the R-allele, could specificly reduce their higher cardiovascular risk by changing dietary habits.

Key words tomato juice – lycopene – antioxidant – lipid peroxidation – paraoxonase – elderly

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List of abbrevitations

CHD coronary heart disease; *dNTP* deoxynucleoside triphosphate; *FRAP* ferric reducing ability of plasma; *PBMC* peripheral blood mononuclear cells; *PCR* polymerase chain reaction; *PON* paraoxonase; *Q* glutamine; *R* arginine; *RFLP* restriction fragment length polymorphism; *TBARS* thiobarbituric acid reactive substances.

Introduction

The oxidative modification of LDL is considered to play a central role in the pathogenesis of atherosclerosis and coronary heart disease (CHD) [1]. Paraoxonase 1 (PON1) is an HDL associated enzyme that belongs to the paraoxonase gene family (PON1, PON2, PON3) and protects LDL from oxidation [2–4]. PON1 may therefore reduce the development of atherosclerosis [5, 6]. An activity polymorphism for PON1 has been shown to be an amino acid substitution glutamine(Q)/arginine(R) at position 192 [7, 8]. The QQ-genotype results in an isoen-

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zyme showing a low activity towards paraoxon (an organophosphate), the isoenzyme corresponding to the RR-genotype exhibits a high activity towards paraoxon. For the PON1 substrate phenylacetate, substrate activity has been shown not to be affected by the PON1–192 genotype [5]. With respect to the PON1–192 polymorphism recent findings indicate that diminished PON1 concentrations and an increased prevalence of the RR-allel is present in populations at increased risk for CHD [9].

Dietary factors such as alcohol [10], pomegranate juice [11] and dietary fat [12–15] have been reported to modulate PON1 activity and may thus have an impact on CHD. It has also been shown by Kleemola et al. [16] that a high vegetable intake is associated with lower PON1 activity and that PON1 activity is negatively correlated with the intake of β-carotene as revealed by a 3-day food record. Data on other carotenoids, e. g. lycopene, were not presented in this study.

Tomatoes and tomato products are the major source for lycopene in the human diet. Tomato consumption resulting in high adipose tissue concentration of lycopene is associated with a reduced risk for myocardial infarction [17], while a low serum lycopene concentration is correlated with an increased incidence of acute coronary events and stroke [18]. Whether lycopene intake and lycopene plasma concentration would affect PON1 activity has not been investigated so far. Therefore, we studied the effect of tomato juice consumption on PON1 activity and other parameters related to oxidative stress in healthy elderly subjects. Furthermore, the PON1–192 genotype has been determined in the volunteers in order to see whether possible treatment effects are related to a distinct PON1–192 genotype.

Material and methods

Subjects

Subjects were participants of the Longitudinal Study in an Aging Population of Giessen (GISELA), Germany, in which the nutritional and health status of free-living elderly people is investigated in yearly intervals. Subjects had to be at least 60 years of age, physically mobile, and available around Giessen for the long term. The GISELA study was initiated in 1994 and in 1997 453 elderly people were enrolled in this investigation. Out of the 453 subjects included in the survey, selection for the participants of the tomato study was based on the following exclusion criteria: smoking, alcohol consumption > 50 g/d, food allergies, cancer, coronary heart disease, chronic inflammatory diseases (rheumatoid arthritis, Crohn disease, colitis ulcerosa), diabetes mellitus, asthma, prescription medication or nonsteroidal anti-inflammatory drugs on a regular basis, vitamin or mineral supplements for the last 3 months, corticosteroid treatment, intake of immunostimulators for the last 4 weeks. Fifty-three elderly subjects (33 females, 20 males) were enrolled after screening. The study was approved by the Ethical Committee of the Department of Medicine, Justus-Liebig-University Giessen and all participants gave their consent in writing.

Study design

Subjects were randomly assigned to control (mineral water) or intervention group (tomato juice). Subjects of the tomato juice group consumed daily 330 mL tomato juice (providing 47.1 mg lycopene and 1.7 mg β-carotene; Schoenenberger, Magstadt, Germany) for a period of 8 weeks. Instead of tomato juice subjects of the control group consumed the same volume as mineral water. Both groups were instructed to drink the tomato juice or water with their main meal. Since the current intake of lycopene in Germany is around 1 mg/d [19] and the tomato juice provided about 40 times more lycopene, subjects were allowed to continue with their regular diet throughout the study period including tomato products in the control group. During the entire intervention period, subjects were asked to protocol any intercurrent diseases or medicine use. Blood samples were drawn after an overnight fast between 7 am and 10:30 am at the Institute of Nutritional Sciences, University of Giessen.

Analytical methods

The "ferric reducing ability of plasma" (FRAP) was used to determine antioxidant activity in plasma [20]. The assay is based on the principle that electron-donating antioxidants can be described as reductants. In this context, the reducing ability of plasma may be refered to as antioxidant activity. The *ex vivo* oxidation of isolated LDL was performed by using a modified method of Esterbauer et al. [21]. The LDL oxidation process was followed by recording the conjugated diene absorption at 234 nm. From the resulting curve, the time interval (minutes) between the intersept of the linear last square slope of the curve with the initial-absorbance axis is defined as the "lag time" and is a measure of the susceptibility of LDL to oxidation. Both methods have been described earlier [22]. Plasma malondialdehyde was measured as thiobarbituric acid reactive substances (TBARS) by HPLC with fluorescence detection [23, 24]. Carotenoids in plasma were measured by reversed-phase HPLC [25]. Serum triacylglycerol, cholesterol and HDL-cholesterol were determined by enzymatic kits (Roche Mannheim, Germany). LDL-cholesterol was calculated by using the "Friedewald"-formula. Plasma pro-

tein thiols were measured with a spectrophotometric method using dithionitrobenzene, while glutathione served as standard [26].

Arylesterase activity of paraoxonase 1

PON1 activity was determined spectrophotometrically using phenylacetate as substrate as described by Gan et al. [27] with minor modifications. The assay mixture contained 5 mmol/L of phenylacetate and 0.9 mmol/L $CaCl_2$ in 20 mmol/L Tris-HCl, pH 8, at 25 °C. The reaction was recorded at 270 nm. Nonenzymatic hydrolysis of phenylacetate was substracted from the total rate of hydrolysis. Results are expressed as U/mL. The E_{270} for the reaction is 1310 mol x L^{-1} cm^{-1} and 1 unit of arylesterase activity is equal to 1 micromole of phenylacetate hydrolyzed per milliliter per minute.

Determination of the paraoxonase 192 genotype

Peripheral blood mononuclear cells (PBMC) were isolated from blood (K-EDTA) by density gradient centrifugation using Histopaque 1077 (Sigma, Deisenhofen, Germany) as described earlier [28]. Genomic DNA was extracted from isolated PBMC using the GenomicPrep Blood DNA Isolation Kit (Amersham, Freiburg, Germany) according to the manufacturers directions. The quantity and quality of prepared genomic DNA were measured at 260/280 nm in a UV/VIS spectrophotometer (Lamda Bio 20, Perkin Elmer, Wellesley, MA, USA). The determination of the paraoxonase 192 (PON1–192) genotype followed a previously published protocol [8] with some minor modifications: The 99 bp target region, which encompasses the polymorphic region of the human PON1 gene, was amplified by polymerase chain reaction (PCR) using specific sense 5′ > TAT TGT TGC TGT GGG ACC TGA G < 3′ (nt 58–79; GenBank Acc. HSPON1EX6) and antisense 5′ > CAC GCT AAA CCC AAA TAC ATC TC (nt 134–156; Acc. HSPON1EX6) primers. The 50 µL reaction mixture contained 1 µg of genomic DNA, 10 nmol of each dNTP, 5 µL of 10x reaction buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris/HCl pH 8.8, 20 mM $MgSO_4$, 1 % Triton X-100), 200 ng of each primer and 0.5 µL VentR DNA polymerase (2 U/µL, New England Biolab, Schwalbach, Germany). After denaturation of DNA at 95 °C for 3 min, the reaction mixture was subjected to 40 PCR cycles (Thermocycler PTC 200; MJ Research Inc., Waltham MA, USA), each cycle comprising denaturation at 94 °C for 60 sec, primer annealing at 55 °C for 30 sec and extension at 72 °C for 60 sec with a final extension time at 72 °C for 5 min. Efficiacy of PCR amplification was checked after electrophoretic separation of a 20 µL aliquot in 3 % agarose and UV-visualization of the amplification product by ethidium bromide staining. Subsequently, a 25 µL aliquot of the PCR product was digested with 5.0 U *Alw1* restriction endonuclease (2 U/µL, New England Biolabs) for 2 h at 37 °C, and the digested products were separated by agarose gelelectrophoresis using 5 % MS500 MoSieve agarose (PeqLab, Erlangen, Germany) in 1x TBE buffer, stained with ethidium bromide and visualized using computer based image analysis (FluorS Imager; Biorad, München, Germany). The 192 Q/R transition creates a unique *Alw1* site in the PCR amplicon. DNA samples homozygous for the 192 Q allele present the original undigested 99 bp PCR product, those homozygous for the 192 R allele present two restriction fragments 66 bp and 33 bp and those heterozygous show the original product of 99 bp plus the restriction fragments of 66 bp and 33 bp. Each genotype was read by two independent observers.

Statistical analysis

Results are given as means ± standard deviation (SD). Baseline characteristics for the PON1 genotype groups (QQ, QR, RR) were analyzed by ANOVA. For comparison of the treatment groups (tomato juice or mineral water), baseline data versus post-treatment data within groups were analyzed by using Students paired t-test or Wilcoxon's rank test for data that were not normally distributed. Normal distribution of the data was analyzed by using the variance ratio test (F-test). Differences between treatment groups were analyzed by using Student's t-test for independent samples (or the Mann-Whitney U test for data that were not normally distributed) on mean pre- to post-intervention differences. Since the RR-genotype was present in 5 subjects only, we merged the QR and RR groups resulting in a R-allele-carrier group. For further analysis the QQ-genotype group was compared with the R-allele-carrier group. Statistical significance was accepted at the $p < 0.05$ level. All statistical calculations were performed with the StatView computer software program (SAS Institute 1998, Cary, NC, USA).

Results

Fifty subjects completed the study; 3 subjects were excluded from the study because they received a drug treatment during the intervention period. No other subjects reported any intercurrent diseases during the study. Subject characteristics are summarized in Table 1.

PON1–192 genotyping revealed 20 subjects carrying the QQ-genotype, 25 QR-genotypes, and 5 RR-genotypes respectively (Table 1). A representative agarose gel image showing the DNA fragments after the *Alw1* digestion is given in Fig. 1. At week 0, there were no differences among the genotype groups with respect to subject characteristics, lipid status (Table 1), LDL-oxida-

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Table 1 Baseline characteristics of study participants1

		Control	Tomato	QQ	QR	RR
n	50	21	29	20	25	5
Sex (F, M)	32, 18	13, 8	19, 10	11, 9	17, 8	4, 1
Age (y)	70±6	70±5	70±6	70±5	70±6	70±3
Body mass index (kg/m^2)	27±3	27±3	26±3	26±3	26±2	30±3*
Triacylglycerol (mg/dl)	101±40	91±28	109±45	97±41	107±41	93±27
Cholesterol (mg/dl)	223±33	220±36	225±32	219±29	219±29	254±55
LDL-Cholesterol (mg/dl)	135±31	132±33	138±31	130±29	134±29	161±47
HDL-Cholesterol (mg/dl)	63±13	65±12	62±14	64±14	61±13	69±6

[1] values are means ± SD; *: $p < 0.05$ for between group differences (QQ, QR, RR)

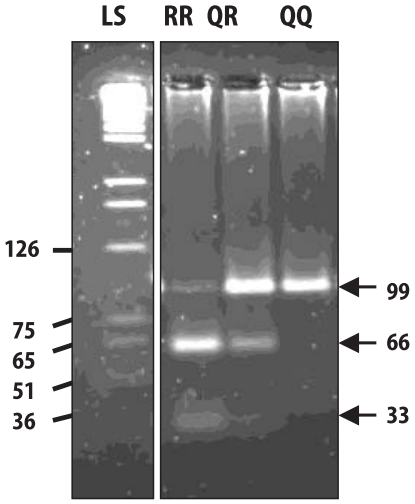

Fig. 1 Determination of the paraoxonase1–192 polymorphism. Representative agarose gel image showing the DNA fragments after the *Alw1* digestion showing the 3 PON1-192 genotypes. *RR* homozygous mutant; *QR* heterozygote; *QQ* homozygous wildtype. Right panel: 33, 66, 99 indicate the size (base pairs) of the digested DNA fragments. Left panel: standard DNA size markers (length standard: LS)

tion, antioxidant status, and PON activity (data not shown). Only BMI was higher in the RR-genotype group at baseline ($p < 0.05$), which resulted from one male with a BMI of 34.

There were no differences in any of the measured parameters between the tomato group and controls at week 0. Tomato juice consumption reduced LDL-oxidation and improved antioxidant status. Lag time ($p < 0.05$) and FRAP ($p < 0.05$) were increased in the tomato group but not in controls (Table 2). In both groups, PON activity was higher after 8 weeks ($p < 0.05$). TBARS, plasma thiols and serum lipids (data not shown) did not change in the tomato and control group, respectively.

In order to see whether the PON1–192 genotype is related to the changes in LDL-oxidation and antioxidant status after tomato juice consumption, we evaluated these parameters in the tomato group with respect to the frequency of the QQ-genotype and the R-allele-carriers (Table 3). Tomato juice consumption increased lag time ($p < 0.05$) and FRAP ($p < 0.05$) and reduced TBARS ($p < 0.05$) in R-allele-carriers. In the QQ-genotype group however, there were no changes regarding LDL-oxidation and antioxidant status. The observed increase in PON activity seems not to be a result of the tomato juice intervention, since PON activity had also increased in the control group (Table 2). In the tomato group, plasma thiols and serum lipids (data not shown) were not different between QQ-genotype and the R-allele-carriers.

The increase in plasma carotenoid concentrations after tomato juice consumption could contribute to the improved antioxidant status and LDL-oxidation. Plasma carotenoid concentrations of the control and tomato

Table 2 Effect of tomato juice consumption on lipid peroxidation and antioxidant status in healthy elderly[1]

	Week 0		Week 8	
	Control	Tomato	Control	Tomato
Lag time (min)	83±12	86±14	82±11	93±14*, [a]
TBARS (µmol/L)	0.70±0.2	0.68±0.2	0.73±0.3	0.66±0.3
PON (U/mL)	102±28	101±29	119±25*	113±32*
FRAP (µmol/L)	887±122	901±224	901±122	948±172*
Thiol groups (µmol/L)	267±53	310±104	291±57	319±113

[1] values are means ± SD; n = 21 (control) or n = 29 (tomato); *TBARS* thiobarbituric acid reactive substances; *PON* paraoxonase-arylesterase activity; *FRAP* ferric reducing ability of plasma; [a] $p < 0.05$ for between group differences (control vs. tomato juice) and * $p < 0.05$ for within group differences (pre-post comparison) after tomato juice consumption

Table 3 Effect of tomato juice consumption and paraoxonase (PON) Q192R polymorphism on lipid peroxidation and antioxidant status in healthy elderly[1]

	Week 0		Week 8	
	QQ	QR/RR	QQ	QR/RR
Lag time (min)	88±19	84±11	94±13	93±15*
TBARS (μmol/L)	0.69±0.2	0.67±0.2	0.72±0.3	0.62±0.2*
PON (U/mL)	99±35	103±26	114±38*	112±28*
FRAP (μmol/L)	990±246	838±190	1009±202	905±138*
Thiols (μmol/L)	319±83	303±119	310±78	326±134

[1] values are means ± SD; n = 12 (QQ) or n = 17 (QR/RR); *TBARS* thiobarbituric acid reactive substances; *PON* paraoxonase-arylesterase activity; *FRAP* ferric reducing ability of plasma; * $p < 0.05$ for within group differences (pre-post comparison) after tomato juice consumption

group have already been published [28]. Table 4 shows the plasma carotenoid concentrations of the tomato group with respect to the PON1 genotype. In both groups, the QQ-genotype and the R-allele-carriers, lycopene and β-carotene increased after tomato juice consumption, while plasma α-carotene, lutein, and β-cryptoxanthin did not change. There were no significant differences in plasma carotenoids between the genotype groups before and after tomato juice consumption. However, there was a tendency for total-lycopene to be higher in the R-allele-carriers ($p = 0.11$).

Discussion

The aim of our study was to investigate the effect of tomato juice consumption on PON1 activity and other parameters related to oxidative stress in healthy elderly subjects. Tomato juice consumption for 8 weeks reduced LDL-oxidation in healthy elderly. This is in line with previous studies showing that in healthy volunteers tomato products protected LDL from oxidation [22, 29]. Additionally, the antioxidant status as measured by the FRAP-assay increased after tomato juice consumption (providing 47.1 mg lycopene/d). Lee et al. [30] also reported an increased antioxidant activity (FRAP) after consumption of tomato products (46 mg lycopene/d) for one week in young volunteers. In contrast, Pellegrini et al. [31] found no effect of tomato puree consumption (7 mg lycopene/d) for two weeks on total antioxidant capacity (TRAP). The type of tomato product (juice, soup, puree) and/or the amount of lycopene supplied may account for the observed differences in antioxidant activity. Lycopene, the major carotenoid in tomatoes, exhibits antioxidant activities in different in vitro systems [32–35] and may be responsible for the observed antioxidant effects. Furthermore, tomatoes contain considerable amounts of polyphenolics and phenolic acids [36, 37], which could also contribute to these effects.

In order to see whether the PON1–192 genotype has an impact on LDL-oxidation and antioxidant status after tomato juice consumption, we analysed the tomato group with respect to the QQ-genotype and the R-allele-carriers. Within the tomato group we found that only in R-allele-carriers tomato juice intervention significantly reduced LDL-oxidation and TBARS, respectively, and increased antioxidant activity (FRAP). This is the first report, that a dietary intervention in humans has an impact on the antioxidant status depending on a distinct PON1–192 genotype. Other groups investigated the ef-

Table 4 Plasma carotenoid concentrations and PON1 Q192R polymorphism in healthy elderly after tomato juice consumption[1]

	Week 0		Week 8	
	QQ	QR/RR	QQ	QR/RR
	(μmol/L)			
Lutein	0.29±0.19	0.34±0.20	0.31±0.19	0.33±0.18
β-Cryptoxanthin	0.17±0.11	0.19±0.16	0.15±0.08	0.17±0.14
total-Lycopene	0.32±0.31	0.22±0.13	0.91±0.34*	1.06±0.39*, [a]
all-trans-Lycopene	0.13±0.05	0.13±0.09	0.48±0.19*	0.59±0.22*
cis-Lycopene	0.19±0.29	0.09±0.05	0.43±0.16*	0.47±0.18*
α-Carotene	0.17±0.11	0.20±0.15	0.20±0.11	0.21±0.08
all-trans-β-Carotene	0.68±0.27	0.79±0.50	0.97±0.23*	1.20±0.59*
cis-β-Carotene	0.05±0.02	0.05±0.02	0.08±0.05*	0.08±0.03*

[1] values are means ± SD; n = 12 (QQ) or n = 17 (QR/RR); [a] $p = 0.11$ for between group differences (QQ vs. QR/RR) and * $p < 0.05$ for within group differences (pre-post comparison) after tomato juice consumption

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fect of pomegranate juice [11], alcoholic beverages [10] and used-cooking fat [13] on arylesterase activity of PON1 in healthy volunteers. While pomegranate juice and alcoholic beverages increased PON activity, used-cooking fat decreased it. Additionally, pomegranate juice reduced LDL oxidation while the used-cooking fat increased LDL oxidation. The authors concluded that the changes in PON activity due to the dietary intervention may reduce the developement of CHD, since low serum PON activity has been reported to be associated with an increased risk for coronary artery disease [5]. Unfortunately, in these dietary intervention studies the PON1–192 polymorphism had not been determined, which would have allowed to see whether the changes in PON activity and LDL oxidation depend on the PON1–192 genotype. In our study, PON activity increased in the control and in the tomato group with no differences regarding the PON1–192 genotype. There were no significant changes in serum HDL concentrations which could account for these changes in PON activity. We suppose, that the observed increase in PON-arylesterase activity may represent a "seasonal" effect during the 8 weeks lasting study period, which we can not explain sofar. Therefore, tomato juice consumption improved antioxidant status in healthy elderly independent of PON activity. However, arylesterase activity of PON1 may not be the adequate method to determine the LDL-protecting properties of PON, since the phenylacetate substrate activity is not affected by the PON1–192 genotype [5]. The enzyme activity related polymorphism for PON1 at position 192 results in an isoenzyme showing a low activity towards paraoxon (QQ-genotype) and an isoenzyme exhibiting a high activity towards paraoxon (RR-genotype), while the lipid hydroperoxide hydrolyzing activity, which is important for the LDL-protecting activity, is low for this isoenzyme [5]. A method for directly measuring the lipid hydroperoxide hydrolyzing activity of PON in human serum is not available so far. Therefore, we prefered phenylacetate as a substrate instead of the toxic paraoxon for PON activity measurements.

The antioxidant status (LDL oxidation, TBARS, FRAP) improved significantly after tomato juice only in the R-allele-carriers, although tomato juice consumption increased plasma carotenoid concentrations in the QQ-genotype group and the R-allele-carriers. Assuming that lycopene from tomato juice is responsible for the improved antioxidant status, differences in plasma lycopene concentrations between the genotype groups could account for the observed differences in antioxidant status. The net increase in total lycopene was higher in R-allele-carriers as compared to the QQ-genotype (QQ: $0.60 \pm 0.38\mu M$ vs. QR/RR: $0.85 \pm 0.39\mu M$). However, these results were not statistically significant ($p = 0.11$). Differences in carotenoid bioavailability among the PON1–192 genotypes, which we can not explain with this study, could contribute to differences in the antioxidant status. We did not assess the concentrations of other antioxidants from tomatoes in the plasma of the study participants and therefore can not discuss their role in improving LDL oxidation and antioxidant activity after tomato juice consumption in healthy elderly.

Recently, Senti et al. [38] investigated the involvement of the PON1–192 polymorphism in the different responses of plasma lipids to physical activity. They found that men with the R-allele need to be physically active to achieve a favorable lipoprotein profile which is similar to that observed in QQ homozygous men. Comparable results related to oleic acid intake have been presented by Tomás et al. [15]. They found that high oleic acid intake was associated with increased HDL cholesterol and PON1 activity only in QR and RR genotypes (R-allele carrier), respectively. From these and from our results we may speculate, that R-allele carrier, who are at a higher risk for CHD as compared to the QQ-genotye, could specifically reduce their risk by changing dietary and life style (exercise) habits.

In conclusion, our data show that tomato juice consumption for 8 weeks improves antioxidant status (LDL oxidation, TBARS, FRAP) in healthy elderly. These changes seem to depend on the PON1–192 polymorphism, since the improvement of the antioxidant status is present in R-allele carriers (QR/RR) only, but not in the QQ wildtype. However, antioxidant status in this study is not related to PON-arylesterase activity. The reason for the differences in the relative increase in plasma lycopene between R-allele carriers and the QQ wildtype has to be investigated in future studies.

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Supplementation of a Low-Carotenoid Diet with Tomato or Carrot Juice Modulates Immune Functions in Healthy Men

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Key Words

Carotenoid · Tomato · Carrot · β-Carotene · Lycopene · Cell-mediated immunity · Natural killer cells · Cytokines

Abstract

Background: β-Carotene has been shown to enhance immune functions in humans. Whether vegetables rich in carotenoids, such as β-carotene or lycopene, modulate immune functions in healthy humans is presently not known. The objective of this study was to investigate the effects of a low-carotenoid diet supplemented with either tomato (providing high amounts of lycopene) or carrot juice (providing high amounts of α- and β-carotene) on immune functions in healthy men. ***Method:*** In a blinded, randomized, cross-over study, male subjects on a low-carotenoid diet consumed 330 ml/day of either tomato juice (37.0 mg/day lycopene) or carrot juice (27.1 mg/day β-carotene and 13.1 mg/day α-carotene) for 2 weeks with a 2-week depletion period after juice intervention. Immune status was assessed by measuring lytic activity of natural killer (NK) cells, secretion of cytokines (IL-2, IL-4, TNFα), and proliferation by activated peripheral blood mononuclear cells. ***Results:*** Juice consumption resulted in relatively fast responses in plasma carotenoid concentrations ($p < 0.0002$) which were not accompanied by concomitant changes in immune functions. For IL-2, NK cell cytotoxicity, and lymphocyte proliferation, maximum responses were observed during depletion periods. The highest production rate was measured only for TNFα at the end of the first intervention period. Juice intervention did not modulate the secretion of IL-4. ***Conclusions:*** Increased plasma carotenoid concentrations after vegetable juice consumption are accompanied by a time-delayed modulation of immune functions in healthy men consuming a low-carotenoid diet.

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Introduction

Diets rich in fruits and vegetables are recommended to maintain overall good health and to prevent chronic diseases. Compounds in this kind of healthy diet may modulate different aspects of immunity. Especially for β-carotene several studies suggest that this carotenoid may stimulate the immune system [1]. Supplementation with pure β-carotene stimulated lymphocyte proliferation in several human intervention studies [2–4]. Lytic activity of natural killer (NK) cells was enhanced in elderly subjects on long-term β-carotene supplementation [5], and after short-term supplementation the percentage of NK cells was significantly increased [6].

Recently, other carotenoids including lycopene and lutein have received more attention. Several studies revealed that a high intake of these carotenoids is associated with a reduced incidence of prostate and lung cancer [7,

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8]. A high intake of tomato and tomato-based products is also consistently associated with a low risk of cancer for a variety of anatomic sites [7]. Numerous potentially immunomodulatory compounds are present in tomatoes with lycopene being the major phytochemical [9]. So far only one study has looked at the immunomodulatory activity of pure lycopene. In contrast to β-carotene, this carotenoid (15 mg/day) did not stimulate monocyte surface molecule expression and tumor necrosis factor-α (TNFα) secretion in a human intervention study [10]. Overall, few data are available on the immunomodulatory activity of the various carotenoids and carotenoid-rich vegetables.

We have recently shown that, based on a low-carotenoid diet, 2 weeks of tomato juice supplementation (330 ml/day providing 40 mg lycopene/day) stimulated lymphocyte proliferation and IL-2 secretion of peripheral blood mononuclear cells (PBMC) in healthy adults [11]. However, in this study only a few immune functions were measured and no washout period after juice consumption was included. In a further study, on the basis of an unrestricted diet, tomato juice supplementation (330 ml/day for 8 weeks) had no effect on various immune functions in healthy elderly subjects [12]. The objective of the present study was to compare, in a cross-over design, the immunomodulatory potential of carrot and tomato juices on immune functions in healthy men consuming a restricted, low-carotenoid diet. Instead of a single-agent study, a whole-diet approach providing active compounds in highly accessible food matrices was used because, in epidemiologic studies, data suggesting a lower cancer risk with a high intake of carotenoid-rich vegetables and fruits were also found on the whole-food level.

Subjects and Methods

Study Design

This study was a randomized cross-over study of two 14-day treatments of tomato juice or carrot juice (330 ml/day, Schoenenberger, Magstadt, Germany). The tomato juice supplement (330 ml/day) provided 37.0 mg lycopene and 1.6 mg β-carotene. Carrot juice supplementation (330 ml/day) delivered 27.1 mg β-carotene and 13.1 mg α-carotene. Treatment periods were preceded by 2-week low-carotenoid periods. After the second treatment period a third 2-week low-carotenoid period followed resulting in a total study period of 10 weeks. In an earlier study we found that a 2-week depletion or supplementation period is long enough to induce significant changes in plasma carotenoid concentrations and in lymphocyte functions [11]. Subjects were told to consume the juices with their main meals. Subjects were not restricted in their daily diet, except that they had to abstain from fruit and vegetables high in carotenoids throughout the whole study period. A list of the fruit and vegetables the subjects were not allowed to eat was provided [13].

Study Population

Twenty-two healthy, non-smoking, non-supplement-taking men were recruited for the study. All subjects were in good medical health as determined by a screening history and medical examination. The study was approved by the Medical Ethical Committee of the Landesärztekammer Baden-Württemberg, and all participants gave their consent in writing.

Isolation of PBMC and Preparation of Serum

Venous blood samples were collected once a week between 7:00 and 9:00 in the morning after the subjects had fasted overnight. Blood collection was done on 2 days with 11 subjects/day. Blood samples were collected into 9-ml tubes containing lithium heparin. Plasma was collected after centrifugation at 1,500 *g* for 10 min at 4°C. PBMC were isolated by density gradient centrifugation. For serum preparation blood was collected and the clotted blood was centrifuged at 1,854 *g* for 10 min at 4°C. Autologous serum (AS) from each subject for the culture of PBMC was heat-inactivated for 30 min at 56°C.

HPLC Analysis of Carotenoids and Retinol in Plasma

Carotenoids and retinol in plasma were analyzed every other week according to a recently described method [14].

Lymphocyte Proliferation

PBMC at 1×10^9 cells/l in medium containing 10% AS were stimulated by the T-cell mitogen concanavalin A (5 mg/l, ConA, Sigma) for 120 h at 37°C. AS was used instead of fetal bovine serum for the culture of PBMC, because it provides PBMC with various plasma carotenoids throughout the length of the cell culture period. In contrast, fetal bovine serum is free of carotenoids [12]. Proliferation was measured as described earlier [11].

Quantification of Cytokine Secretion

PBMC at 1×10^9 cells/l were cultured in medium containing 10% AS and stimulated by 5 mg/l ConA for 24 h at 37°C (IL-2, IL-4) or by 1 μg/l lipopolysaccharide (Difco, Augsburg, Germany) for 24 h at 37°C (TNFα). Cell-free supernatants were collected and stored at –80°C until analysis. IL-2, IL-4, and TNFα were measured by sandwich-ELISAs as described earlier [12].

Lytic Activity of NK Cells

The lytic activity of NK cells as part of PBMC against K562 target cells (PBMC:K562 ratios 25:1, 12.5:1, 6.25:1) was measured with a recently described flow cytometric method [15] and calculated as the percentage of dead target cells in the test samples minus the percentage of dead target cells in the control samples without effector cells.

Statistical Analyses

We used multi-way repeated measures analysis of variance (ANOVA) to analyze changes of the various immune parameters. Treatment (tomato or carrot juice), period (first juice treatment or second juice treatment), and diet sequence (tomato/carrot vs. carrot/tomato) were included as main effects and change in immune parameters over the period (week 2 vs. week 4 and week 6 vs. week 8) as dependent variables. Within-subject effects and between-subject effects were also included in the analyses. When significant diet sequence effects were observed by repeated-measures ANOVA, differences in the changes over the periods were analyzed by paired Stu-

Table 1. Plasma carotenoid concentrations (μmol/l) of subjects consuming a low carotenoid diet supplemented with tomato or carrot juice (mean ± SD; n = 11/treatment group)

Study period Week	Baseline 0	Depletion 2	Juice 4	Depletion 6	Juice 8	Depletion 10
β-Carotene						
Tomato/carrot	0.37 ± 0.32	0.25 ± 0.20	0.21 ± 0.16	0.20 ± 0.14	1.00 ± 0.53*	0.45 ± 0.29
Carrot/tomato	0.45 ± 0.45	0.27 ± 0.21	1.18 ± 0.44*	0.49 ± 0.14	0.41 ± 0.17	0.25 ± 0.10
α-Carotene						
Tomato/carrot	0.09 ± 0.09	0.06 ± 0.06	0.05 ± 0.04	0.05 ± 0.04	0.53 ± 0.22*	0.27 ± 0.10
Carrot/tomato	0.08 ± 0.06	0.06 ± 0.04	0.61 ± 0.26*	0.27 ± 0.11	0.17 ± 0.07	0.10 ± 0.03
Lycopene						
Tomato/carrot	0.45 ± 0.29	0.21 ± 0.12	0.52 ± 0.20*	0.21 ± 0.09	0.16 ± 0.13	0.10 ± 0.08
Carrot/tomato	0.55 ± 0.41	0.23 ± 0.11	0.14 ± 0.07	0.12 ± 0.10	0.48 ± 0.21*	0.18 ± 0.07
Lutein						
Tomato/carrot	0.31 ± 0.13	0.25 ± 0.06	0.24 ± 0.10	0.23 ± 0.07	0.31 ± 0.06	0.23 ± 0.05
Carrot/tomato	0.31 ± 0.09	0.27 ± 0.07	0.30 ± 0.08	0.26 ± 0.06	0.26 ± 0.07	0.24 ± 0.05
β-Cryptoxanthin						
Tomato/carrot	0.11 ± 0.07	0.08 ± 0.04	0.06 ± 0.04*	0.06 ± 0.04	0.06 ± 0.04	0.05 ± 0.03
Carrot/tomato	0.16 ± 0.11	0.12 ± 0.06	0.09 ± 0.04*	0.09 ± 0.04	0.07 ± 0.03	0.06 ± 0.03

* Significantly different from week 2 (juice period 1) and week 6 (juice period 2) determined by ANOVA: $p < 0.0002$.

dent's t test using the data from week 2 as the baseline value. All statistical calculations were performed with PROC GLM in SAS Software (version 6.12) and with the StatView program (SAS Institute 1998, Cary, N.C., USA).

Results

All subjects (aged 28.7 ± 5.9 years, body mass index 23.1 ± 2.0 kg/m^2) complied with the study protocol and completed both dietary treatments. Plasma carotenoid concentrations were determined including all trans and cis configurations. Results for β-carotene and lycopene are shown as total β-carotene and total lycopene. There was no significant difference in plasma carotenoid concentrations between groups at baseline (table 1). Carrot juice consumption significantly increased plasma concentrations of β- and α-carotene, and tomato juice consumption resulted in elevated lycopene concentrations (table 1). While lutein concentrations did not change significantly during juice consumption, β-cryptoxanthin concentrations were reduced at the end of the first juice supplementation period (table 1). The absolute increase in plasma carotenoid concentrations did not differ between the 2 groups (data not shown). Therefore, the sequence of the juice intervention had no effect on the carotenoid bioavailability from vegetable juices. Plasma retinol concentrations were measured to control whether juice-derived provitamin A carotenoids increased plasma retinol concentrations. Juice supplementations did not change plasma retinol concentrations significantly (data not shown).

In general, crossover designs can induce 2 types of bias as a result of diet sequence and period effects. We observed significant diet sequence effects for lymphocyte proliferation, lytic activity of NK cells and for secretion of TNFα. Therefore, we analyzed changes in immune functions before and after juice supplementation (period 1: week 2 vs. week 4; period 2: week 6 vs. week 8) by paired Student's t test. Proliferation of PBMC cultured in AS did not differ between both groups at baseline. Compared with the end of the first low-carotenoid period, juice intervention did not modulate proliferative responsiveness of PBMC (table 2). One week after the end of the first juice supplementation period until the end of the whole intervention period lymphocyte proliferation was significantly enhanced in both groups (table 2).

The lytic activity of NK cells at baseline and throughout the study period was comparable between groups. During the first supplementation period and the following depletion period lytic activity increased significantly (table 2). While during the second juice supplementation period lytic activity first decreased to the baseline level, lytic activity was again significantly enhanced at the end of the second juice supplementation (carrot juice) 1 week after tomato juice supplementation (table 2). As a further control, the cytotoxicity of cryopreserved NK cells from 1

Table 2. Proliferation of peripheral blood mononuclear cells (activated with ConA 5 mg/l for 120 h at 37°C) and lytic activity of NK cells from subjects consuming a low-carotenoid diet supplemented with tomato juice (weeks 2–4 or 6–8) and carrot juice (weeks 2–4 or 6–8; mean ± SD; n = 11/treatment group)

Study period	Depletion		Juice		Depletion		Juice		Depletion		
Week	0	1	2	3	4	5	6	7	8	9	10
Lymphocyte proliferation											
Tomato/carrot	1.0 ± 0.2	1.2 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	2.1 ± 0.3***	2.0 ± 0.2***	1.8 ± 0.2***	1.5 ± 0.3*	1.5 ± 0.4*	1.6 ± 0.2**
Carrot/tomato	1.0 ± 0.2	1.3 ± 0.2	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	2.0 ± 0.5***	1.9 ± 0.4***	1.7 ± 0.4***	1.5 ± 0.3	1.5 ± 0.4	1.5 ± 0.3*
NK cell activity											
Tomato/carrot	55 ± 5	65 ± 6	52 ± 5	67 ± 6*	65 ± 5*	66 ± 6*	62 ± 5*	51 ± 6	63 ± 5*	68 ± 4*	59 ± 4
Carrot/tomato	51 ± 4	61 ± 5	56 ± 3	65 ± 5	61 ± 3	70 ± 4**	65 ± 5	62 ± 4	55 ± 4	70 ± 4**	60 ± 3

Proliferation is presented as measured absorbance at 450 nm minus absorbance at 650 nm; lytic activity of natural killer cells with an effector:target ratio of 25:1.

Significantly different from week 2 as determined by Student's t test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

individual not participating in the study was quantitated on each day that the NK cell activity of study subjects was measured. The mean NK cell activity was 54.1 ± 3.2 (a total of 21 measurements) and the coefficient of variation was 5.9%.

IL-2 secretion was significantly affected by juice supplementation, again without any differences between carrot and tomato juice (fig. 1). The first intervention period resulted in significantly higher IL-2 secretion in those subjects consuming carrot juice (fig. 1). One week later, both juice supplementations resulted in elevated IL-2 secretion. Carotenoid depletion after the first juice supplementation resulted in reduced IL-2 secretion. The lowest IL-2 concentration was measured when subjects were already drinking the second juice for 1 week. Two weeks after the second juice supplementation had stopped, IL-2 secretion was again significantly increased.

In contrast to IL-2, the secretion of IL-4 by ConA-activated T lymphocytes was not affected by either juice supplementation (data not shown). TNFα was measured as a cytokine primarily produced by human monocytes. Supplementation with either juice significantly enhanced TNFα secretion (weeks 2–4) and, during the subsequent low-carotenoid period, a significantly reduced TNFα secretion was again observed (fig. 2).

Discussion

The major finding of the present study is that diets low or high in carotenoids result in relatively fast responses in plasma carotenoid concentrations, which are not accompanied by concomitant changes in immune functions. The reason for this time delay is currently not known. Assessing the time course of intracellular carotenoid accumulation in PBMC in future intervention studies may help to reveal this issue. Although total carotenoid intake was not assessed in the present study, data from a similar study indicated that the study subjects on such a low-carotenoid diet consumed about 1.3 mg/day of total carotenoids, which is far below the average carotenoid intake of 5.6 mg/day for this age group [11]. The low carotenoid intake in the present study was associated with a comparable reduction in plasma carotenoid concentrations as reported in our previous study. A provitamin A effect of the juice supplementation can be excluded because plasma retinol levels did not change after juice supplementation. No differences between both juices were observed, although only carrot juice supplementation provided high quantities of carotenoids with provitamin-A activity.

Lymphocyte proliferation is a common immune parameter which has been measured in several studies investigating the immunomodulatory effects of β-carotene [2–4, 16, 17]. These studies reported conflicting data, partly because of differences in the dosage and duration of carotenoid supplementation, and the use of carotenoid depletion periods. In the present study, juice supplementation did not affect proliferative responsiveness of PBMC during the first juice supplementation period. However, 1 week after juice supplementation had been stopped, lymphocyte proliferation was significantly increased ($p < 0.001$). Probably as a result of the low-carotenoid diet and repletion with juices, carotenoid depletion affects the regulatory processes of PBMC proliferation, and it may take several days for this to result in enhanced proliferation. In other studies longer time periods be-

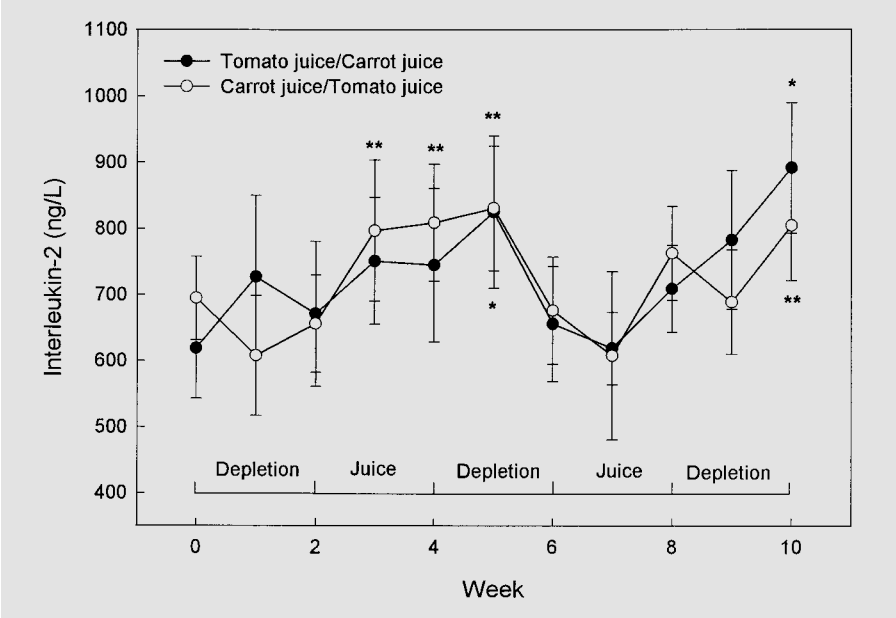

Fig. 1. Interleukin-2 production of activated peripheral blood mononuclear cells (5 mg/l ConA, 24 h at 37 °C) from subjects consuming a low-carotenoid diet supplemented with tomato juice (weeks 2–4 or 6–8) and carrot juice (weeks 2–4 or 6–8). Values are mean ± SEM, n = 11/treatment group. Significantly different from week 2 as determined by Student's t test: * $p < 0.05$; ** $p < 0.01$.

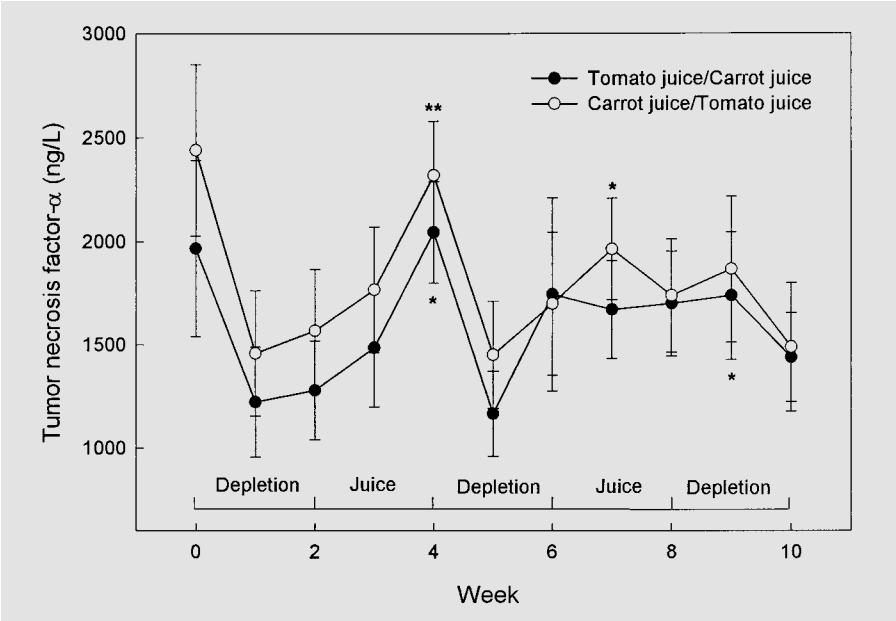

Fig. 2. TNF-α production of activated peripheral blood mononuclear cells (1 μg/l LPS, 24 h at 37 °C) from subjects consuming a low-carotenoid diet supplemented with tomato juice (weeks 2–4 or 6–8) and carrot juice (weeks 2–4 or 6–8). Values are mean ± SEM, n = 11/treatment group. Significantly different from week 2 as determined by Student's t test: * $p < 0.05$; ** $p < 0.01$.

tween dietary intervention and measurement of lymphocyte proliferation have been used [2, 4], which may have masked a potential time delay in this response. The present results are supported by data from another study which demonstrated that, after consuming a low-carotenoid diet for 60 days, supplementation with a carotenoid complex from vegetables enhanced mitogenic proliferative responsiveness of blood lymphocytes in women [4]. In contrast, the same mixed carotenoid supplement had no significant effect on mitogenic T-cell proliferation in a free-living group of elderly women with normal plasma carotenoid profiles at baseline [18].

A stimulatory effect of β-carotene supplementation on NK cell lytic activity has so far only been demonstrated in elderly men on long-term β-carotene supplementation [5] and in healthy adults supplemented with 30 mg/day β-carotene for 3 months [19]. In the present study, supplementation with either vegetable juice resulted in significantly enhanced NK cell lytic activity at the end of the juice supplementation periods, specifically 1 week after

juice supplementations had been stopped. In the studies by Santos et al. [5, 16], the β-carotene plasma concentrations in the placebo groups were around 0.6 μmol/l, but in the present study the concentration was 0.26 ± 0.21 μmol/l at the end of the first depletion period. Again, the low plasma carotenoid concentrations at baseline and the supplementation with a carotenoid mixture from food instead of supplementation with single carotenoids may be relevant for the differences between these studies. So far no study has investigated the effect of pure lycopene or α-carotene on NK cell lytic activity. The low coefficient of variation (5.9%) for this type of assay argues against the observed differences being due to methodological variation.

The lytic activity of NK cells can be regulated by different cytokines. IL-2 has been shown to be a potent stimulatory factor for NK cells increasing lytic activity [20]. In the present study, juice supplementation significantly increased IL-2 secretion by activated PBMC, with no significant differences between the types of vegetables. Again, a time delay of 1–2 weeks between intervention and the maximum responding change in IL-2 secretion was obvious. Peak IL-2 secretions after the first juice supplementation were observed at the same time point (week 5) as the maximum NK cell lytic activity and lymphocyte proliferative responsiveness. In an earlier study tomato juice supplementation to a low-carotenoid diet also resulted in an enhanced IL-2 response [11].

In contrast to IL-2 which is primarily produced by T-helper (TH) 1 lymphocytes, IL-4 is mainly produced by TH2 lymphocytes [21]. The intervention with the low-carotenoid diet as well as the juice supplementations did not change IL-4 secretion significantly, suggesting that TH2 lymphocytes are not affected by this type of dietary intervention.

Previous studies have shown that β-carotene supplementation resulted in enhanced TNFα secretion in humans [2, 22, 23]. In in vitro studies with human PBMC we have demonstrated that β-carotene incorporated into liposomes at physiologic concentrations also stimulated TNFα secretion [24]. The present study clearly shows that both juices during the first supplementation period significantly increased TNFα secretion by mitogen-activated monocytes.

The observed differences in changes in immune functions between supplementation periods 1 and 2 may be related to the fact that during the whole study period subjects were on a low-carotenoid diet. Juice supplementation provided the subjects with only lycopene and β-carotene (tomato) or α- and β-carotene (carrot), but carotenoids such as lutein and β-cryptoxanthin were not provided with the vegetable juices. As a result plasma β-cryptoxanthin concentrations decreased during the first juice supplementation period. Therefore, the degree of carotenoid depletion in tissues such as immune cells may have been higher during the second intervention period.

The underlying mechanisms of juice-induced immunomodulation are currently not known. The antioxidative effects of carotenoids might reduce the amount of free radical generation protecting membrane lipids in PBMC from oxidation [25], which might result in enhanced immune functions. In an earlier study we were able to show that tomato juice supplementation to healthy adults on a low-carotenoid diet significantly reduced low-density lipoprotein oxidation [26], although the level of oxidative DNA damage in PBMC was not affected [27]. In vitro studies also did not reveal a close relationship between modulation of TH lymphocyte function and antioxidant activity of carotenoids [28].

A major limitation of our study is the lack of an appropriate placebo group. Normally, studies with a cross-over design do not require an additional placebo group. Due to the observed time delay in the immunological changes in the present study, such a placebo group would have been helpful for the interpretation of the results. Based on the outcome of this study, it is difficult to determine whether the observed effects during weeks 3–5 were truly related to the juice supplementation during weeks 2–4 or were related to confounding factors. However, the consistent pattern of immune responses with different cell types such as monocytes, TH1 lymphocytes, and NK cells, all peaking within 1 week after stopping the juice intervention, suggests a real effect. A study with similar design assessing the same immune parameters was run immediately after the present carotenoid study. Compared to baseline, no significant changes for control and treatment group during a 12-week period were found [29]. This argues against the idea that the changes in immune functions measured in our study were a chance finding and not related to carotenoid depletion and juice supplementation.

From our earlier studies [11, 30] we know that washout or depletion periods result in either no effect or in decreased lymphocyte proliferation and cytokine production. Therefore, it can be excluded that the observed increase in cytokine production or NK cell activity in the present study was due to the washout or depletion period. In addition, similar effects on immune functions with carotenoid depletion and carotenoid supplementation have been reported by others [4, 18].

In conclusion, the present results suggest that carotenoid-rich vegetables affect the immune system in sub-

jects consuming a low-carotenoid diet. Clearly, no significant differences were observed between supplementation with tomato or carrot juice. This may indicate that either different carotenoids have similar effects on the immune system or compounds other than carotenoids, which occur in both types of vegetables, may be responsible for the effects observed. One example could be the hydroxycinnamic acids [9], which are taken up in substantial quantities with tomatoes and carrots [31].

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Diet and Ethanol Modulate Immune Responses in Young C57BL/6 Mice

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Chronic ethanol (ETOH) ingestion adversely affects the immunocompetence of alcohol abusers. ETOH directly impairs host defense mechanisms and indirectly modulates immunocompetence by interfering with the nutritional status of the alcoholic. It is not clear from the current literature, however, to what extent ETOH, nutritional status, or the combination of the two factors modulates immune mechanisms in chronic alcoholics. To date, most animal studies investigating the immunotoxicity of ETOH have neglected the dietary factors, which may have masked additional immunotoxic effects of ETOH. To examine these dietary factors, we fed mice three liquid ETOH diets with different dietary sufficiencies for 7 weeks and investigated various immune responses. Spleen cell number and secretions of immunoreactive interleukin-2 and tumor necrosis factor were totally independent of the diet, being affected only by ETOH. Body, spleen, and thymus weights, interferon-γ secretion, and natural killer cell and phagocytic activities were modulated by ETOH as well as by diet. Natural killer cell and phagocytic activities were also directly affected by the nutritional quality of the diet. These results suggest that animal diets used in experimental studies of ETOH-induced immunomodulation must be planned and controlled carefully in order to single out the direct effects that ETOH has on the host defense system.

Key Words: Ethanol, Nutritional Status, Animal Diets, Immunocompetence, Mice.

RECENT STUDIES HAVE demonstrated that chronic ethanol (ETOH) ingestion adversely affects immunocompetence and predisposes alcohol abusers to an increased risk of infections.[1] Direct immunotoxic effects of ETOH include the impairment of mechanical host defenses, granulocytopoiesis, chemotaxis, phagocytosis, and cytotoxicity. Additionally, alcohol abuse interferes with the nutritional status of the user, further modulating immunocompetence.[2] Surveys have shown that as many as 75% of chronic alcoholics do not consume the recommended daily allowance of vitamins A, C, thiamin, and folic acid.[3-6] Chronic alcohol abuse also decreases the bioavailability of various nutrients.[7] Consequently, a combination of the direct immunotoxic effects of ETOH and the immunosuppressive effects of malnutrition result in impaired host defenses in chronic alcohol abusers.

Data about the direct immunotoxic effects of ETOH were primarily generated by studies using rats or mice, where ETOH is incorporated into a liquid diet.[8] Liquid diets containing ETOH were introduced by Lieber-DeCarli (LD)[9] to study the pathological hepatic conditions associated with alcohol abuse. Because of their convenience and their success in achieving high blood ethanol levels (BEL), they are now used by many researchers interested in any pathological condition associated with alcohol abuse, including immunotoxicity.[10-13]

Analysis of the composition of the basic LD diet[8] reveals that 35% of the total energy is provided by plant fat containing a high percentage of monounsaturated fatty acid. The LD diet contains 45 times more vitamin A than recommended for the growth of mice by the National Research Council, 10 times more vitamin D, 5 times more vitamin E, and 6 times more pyridoxine.[14] While a surplus of vitamins 2 to 3 times the recommended level may be necessary to balance any vitamin losses that might occur during preparation and storage of the diet, concentrations exceeding 3 times the requirement may obscure experimental findings,[15] and certainly do not accurately reflect the nutrient consumption of human alcohol abusers. Liver stores of vitamin A, for example, are depleted by chronic alcohol abuse[16], which can affect immunocompetence in various ways.[17-19] Therefore, by using the standard LD diet to study ETOH-induced immunomodulation in experimental studies, one might inadvertently exclude the effects of ETOH-induced malnutrition on the host defense. To define the impact of nutrition on the immunomodulatory effects of ETOH, we compared the immunomodulatory effects of the LD-ETOH and two other ETOH-containing liquid diets. The nutrient composition of one diet was modeled on the requirements of growing mice, and the other diet was based on a 40% reduction in these same requirements to more closely resemble the nutrient intake of chronic alcohol abusers. Because ETOH can affect each immunocompetent cell, we investigated the modulatory effects of ETOH on various immune responses.

MATERIALS AND METHODS

Animals and Diets

Female C57BL/6 mice at 3–5 weeks of age were obtained from Charles River Laboratories, Inc. (Wilmington, DE), and were kept on a 12-hour

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dark-light cycle in a temperature-controlled environment (20–22°C). Mice were maintained for 2 weeks in our facility on a nonpurified diet (Teklad, Madison, WI) and water ad libitum. Animals were then assigned randomly to one of the three liquid diet groups, each consisting of three subgroups. Each liquid diet was fed as a liquid ETOH diet (30% of the energy derived by ETOH) and an isocaloric control diet (in which maltose-dextrin substitutes for the energy contributed by ETOH in the ETOH diet), with the mice having free access to each of these diets. Because mice typically consume less food if ETOH is present in the diet, another subgroup was pair-fed with a control diet against the ETOH mice of the same liquid diet group. These mice were restricted to the same amount of food as the mean amount eaten by the ETOH mice. All dietary groups started with 10 mice per group except 100% NRC group fed ETOH, which had 15 mice. Mice were fed once a day between 0800 and 1000 hr and housed five to a cage. ETOH was introduced into the diet over a period of 8 days to allow the mice to adjust to ETOH. Food was changed daily to minimize ETOH evaporation, and food consumption per cage was also measured daily. All diets were prepared fresh every other day and stored in the dark at 4°C. The protocol was approved by the Animal Care Committee of the University of Arizona College of Medicine.

Group 1 was fed the LD liquid diet (no. 710260, Dyets, Bethlehem, PA) containing 30% of energy as ETOH, 17% as carbohydrates, 18% as protein, and 35% as fat. Groups 2 and 3 were fed a LD-type liquid ETOH diet (no. 710279 and 710280, Dyets), in which the protein content was reduced to 12.5% according to the recommendations of the National Research Council (NRC) for the growth of mice[14] (these diets are hereafter called 100% NRC and 60% NRC). The carbohydrate content in these diets was increased to 27.5%, and the fat content was reduced from 35% of the energy in the LD diet to 30%.

The vitamin and mineral contents of diet 100% NRC were set according to the recommendations of the NRC (1978) for the growth and reproduction of mice, as no information was available for growth alone (Table 1). Since the vitamin E content is related to the fat content of the diet, and the NRC recommendation for this vitamin is based on a diet providing 15% of energy as fat, its concentration in our diet (30% of energy as fat) was increased 100%. A recent American Institute of Nutrition workshop[20] recommended lowering the manganese level of diets to 10 mg/kg, which we followed in the 100% NRC diet. As no recommendation is given by the NRC for the selenium content of mouse diets, we included selenium at a concentration recommended by the NRC for rat diets. The 60% NRC diet contained 60% of the vitamin and mineral contents of the 100% NRC diet (Table 1).

The study lasted 7 weeks, after which the mice were killed by ether anesthesia. Blood was collected from the auxiliary vein, and serum was stored at −70°C. The spleen and thymus were removed immediately.

Preparation of Spleen Cells

Spleen cell suspension was made by gently teasing the spleen with forceps in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT). Red blood cells were lyzed by ammonium chloride. The number of viable cells was determined by trypan blue exclusion, and the cells were adjusted to give the desired viable cell concentration.

Stimulation of Spleen Cells for Cytokine Secretion

The cell number was adjusted to 1×10^{10}/liter, and 0.1 ml of cell suspension was added to each well of a 96-well microplate. Total spleen cells after removal of red blood cells were used for cytokine assays, because cell to cell interactions are important factors in the regulation of cytokine secretion. For the induction of interleukin-2 (IL-2) or interferon-γ (IFN), 0.1 ml of Concanavalin A (4 or 10 mg/liter; Sigma, St. Louis, MO), and for the induction of tumor necrosis factor-α (TNF), 0.1 ml of lipopolysaccharide (2 mg/liter, extracted from *E. coli* 0111:B4, Difco, Detroit, MI) were added to each well. Residual endotoxin in the medium and on glassware was not removed. After 24 hr (TNF) and 48 hr (IL-2, IFN), supernatant fluids were collected and stored at −70°C for assay.

Table 1. Ingredients of Control Diets

	LD (g/L or g/4.18 mJ)	100% NRC	60% NRC
Casein	41.4	29.9*	29.9*
DL-Methionine	0.3	0.2	0.2
L-Cysteine	0.5	0	0
Corn oil	8.5	7.5	7.5
Safflower oil	2.7	7.5	7.5
Olive oil	28.4†	0	0
Lard	0	18.0	18.0
Maltose-dextrin	115.2	139.6	139.6
Cellulose	10.0	10.0	10.0
Xanthan gum	3.0	3.0	3.0
Choline bitartrate	0.5	0.3	0.3
Vitamin mix	2.5‡	2.5§	1.5¶
Mineral mix	8.7‡	6.0‖	3.6‖

* Vitamin free.

† The fatty acid pattern for the LD diet was 16.2% saturated, 67.8% monounsaturated, and 15.9% polyunsaturated fatty acids. For the NRC diets, it was 31.4% saturated, 35.9% monounsaturated, and 32.7% polyunsaturated fatty acids.

‡ For information about vitamin and mineral content, see references (Lieber and DeCarli 1989).

§ Vitamin mix provided in milligrams per 4.18 mJ of diet (except as noted): thiamin-HCl, 0.75; riboflavin, 1.05; pyridoxine-HCl, 0.26; nicotinic acid, 2.63 μg; calcium pantothenate, 2.1; folic acid, 0.13; biotin, 0.05; vitamin B-12, 2.63 μg; retinyl palmitate, 39.6 μg; cholecalciferol, 1.0 μg; α-tocopherol acetate, 10.5; menadione sodium bisulfite, 0.79.

¶ The 60% NRC diet provided all vitamins at a concentration which was 60% of that provided by the 100% NRC diet.

‖ Mineral mix provided in grams per kilogram of mix: calcium phosphate, dibasic, 595.64; potassium phosphate, monobasic, 175.10; sodium chloride, 36.27; sodium bicarbonate, 28.25; potassium sulfate, 83.22; magnesium oxide, 36.47; manganous sulfate-H_2O, 1.35; ferrous sulfate-$7H_2O$, 5.46; zinc carbonate, 2.52; cupric carbonate, 0.34; potassium iodate, 0.02; sodium selenite, 0.01; chromium potassium sulfate-$12H_2O$, 0.85. The 60% NRC diet provided all numerals at a concentration which was 60% of that provided by the 100% NRC diet.

IFN Assay

IFN secretion was quantitated by an enzyme-linked immunosorbent assay (ELISA), as described previously.[21] In earlier experiments, we determined that our ELISA detected only biologically active IFN. Microtiter plates (Immulon II, Dynatech, McLean, VA) were coated with 50 μl of a monoclonal antimurine IFN (Lee Biomolecular, San Diego, CA) diluted 1:100 with carbonate buffer, pH 9.6, and stored at 4°C overnight. Microplates were washed with 0.1 mol/liter phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween). Then, 50 μl of IFN standards (recombinant murine IFN, Genzyme, Boston, MA) or 50 μl of supernatant were added to the wells and incubated for 2 hours at 37°C. After washing with PBS-Tween, 50 μl of a rabbit antimouse IFN polyclonal antiserum (a gift from Philip Scuderi, Arizona Cancer Center, University of Arizona) diluted 1:100 was added. After 2 hr of incubation at 37°C and washing with PBS-Tween, 50 μl of a peroxidase-conjugated goat antirabbit immunoglobulin specific antiserum (American Qualex, La Mirada, CA) diluted 1:5000 were added, and the plate was incubated for another hour. Finally, after washing three times with PBS-Tween and once with PBS, the peroxidase substrate 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Sigma) was added, and optical density readings were made using a Titertek multiscan (Flow Labs, McLean, VA) with a 405-mm filter 30 min after substrate addition. The quantity of IFN in culture supernatants was determined by comparison with a set of standards made with recombinant IFN. The limit of detection was at least 200 ng/l, 20 pg/well.

TNF Assay

Secretion of immunoreactive TNF was quantitated by an ELISA as described previously,[22] using a procedure similar to that used in the IFN assay. The monoclonal antibody antimurine TNFα/β (Genzyme) was used at a dilution of 1:500. Recombinant murine TNFα was purchased from Genzyme. The rabbit antimouse TNFα polyclonal antiserum (a

gift from Philip Scuderi) was used at a dilution of 1:167. The standard range was 0.156 to 10 μg/liter with a detection limit of 10 pg/well.

IL-2 Assay

Immunoreactive IL-2 was quantitated by an ELISA. The monoclonal antibody antimurine IL-2 (Genzyme) was used at a dilution of 1:500. Recombinant IL-2 and rabbit antimouse polyclonal antiserum were purchased from Collaborative Research, Inc. (Waltham, MA). The polyclonal antiserum was used at a dilution of 1:2000. The standard range was 0.3 to 20 μg/liter with a detection limit of 30 pg/well.

Natural Killer (NK) Cell Assay

NK cell activity was measured in a 4-hr chromium-51 (Cr-51) release assay against YAC-1 target cells, as described previously.[23] Target cells (1×10^7) were incubated at 37°C for 1 hr in medium containing 5.6 MBg of sodium chromate-51 (New England Nuclear, Boston, MA). Tumor cells were washed to remove unbound radiolabel, resuspended in compiete medium, and added to each well of a 96-well U-bottom microtiter plate (1×10^5 cells/well). Quadruplicate cultures were set up for each individual mouse. Spleen cells (effector cells) were diluted in complete medium and mixed with the target cells so that final effector:target ratios of 100:1, 50:1, and 25:1 were achieved. The plates were centrifuged (180 × *g*) for 2 min and incubated for 4 hr at 37°C. Controls included spontaneous release of Cr-51 from tumor cells alone. Maximum release was determined by measuring Cr-51 release from labeled target cells treated with 100 μl Nonidet P-40 (10 g/L). Then, 100 μl of each aliquot were removed from the supernatant, and samples were counted in a γ-counter. The percent specific lysis was calculated as follows: [(experimental release − spontaneous release) × 100]/maximum release − spontaneous release.

Phagocytosis

The phagocytic activity of spleen cells was determined by flow cytometric quantitation with fluorescent microspheres, as described previously.[24] Spleen cells (1×10^{10}/L) were incubated with 5×10^7 fluorescent latex particles (Fluoresbrite, 1.34 μm diameter, Polysciences, Inc., Warrington, PA) for 2 hr in polypropylene tubes in a horizontal position in a 37°C shaking water bath to achieve optimal mixing between particles and cells. Excess latex particles were removed by layering the reaction mixture over 3 ml of newborn bovine serum, followed by centrifugation (150 × *g*) for 10 min. The supernatant with free latex particles was aspirated, and the cells were washed twice. Extensive washing removed beads associated with cells that were not internalized. Finally, cells were resuspended in complete medium containing 10% dimethylsulfoxide and frozen at −20°C. For flow cytometric analysis, samples were thawed and stained with 50 mg/liter propidium iodide (Sigma). Thawing resulted in only minor damage to the cells, with fewer than 1% of all cells releasing beads. To determine the population's involvement in phagocytosis, an ungated collection of all nucleated cells was taken and analyzed to find the percentage of cells containing one, two, or more than two latex particles. To confirm internalization of beads, samples were analyzed by transmission electron microscopy, with essentially all internalized.

Vitamin A Determination

Hepatic concentrations of total vitamin A were determined by the fluorometric method. Briefly, about 0.1 g of liver was homogenized in 1 ml of water and 1 ml of ethanol, and vitamin A was extracted with 5.0 ml of *n*-hexane by shaking for 1 min and centrifugation at 3000 rpm. The *n*-hexane layer was removed. The concentration of hepatic vitamin A level in this extract was determined as described previously.[25]

Blood Ethanol Level

To determine the BEL after 4 weeks we used a separate group of mice, because we wanted to exclude any risk of infection in the study mice that might be caused by collecting blood from the tail vein. BELs were determined by a gas chromatographic method[26] on blood collected 5 and 20 hr after fresh food was given. BEL were obtained by injecting 0.5 μl of serum into a glass wool plug on a Porpack Q column (Alltech Associates, Deerfield, IL), which was maintained at 180°C. BELs were then measured by comparing individual peak areas to those developed by injecting various amounts of a standard solution containing known amounts of ETOH into an identical column.

Statistical Analysis

Data are presented as the mean ± SD for each group. Data were analyzed using the one-way analysis of variance, followed by a multiple range test with Statistical Package for Social Sciences.[27] The probability level (p) at which differences were considered statistically significant was less than 0.05.

RESULTS

BEL

BEL were measured 5 and 20 hr after fresh food was given. The results show that the BEL at both time points was below the level used as a cut-off point for intoxication in humans (17 mmol/liter; Table 2). No significant difference among the three diets was observed.

Mortality

There was no mortality among mice with free or restricted access to the control diets, with the exception of one mouse in the pair-fed 60% NRC group. There was no mortality observed in the LD-ETOH group. In the 100% NRC-ETOH and 60% NRC-ETOH groups, however, mice started to die 4 weeks into the experiment. By the end of the experiment, only 40% of the mice survived in each of those groups. Gross pathology performed on some of these animals did not identify organ damage as a possible cause of death.

Food Intake and Body Weight

Food intake, expressed as energy consumed per mouse per day, was not significantly different in the three dietary groups (Table 3). Mice with free access to control diets consumed less energy than mice fed ETOH-containing diets. ETOH intake did not differ among the dietary groups (Table 3). The body weights of mice with free access to the control diets were nearly identical, while body weights of mice fed ETOH-containing or energy-restricted control diets were significantly lower than those of controls. Despite a similar ETOH intake, mice fed the LD-ETOH diet achieved a significantly higher body weight

Table 2. Blood ETOH Concentrations in Mice Fed Liquid ETOH Diets

Time Postfeeding (hr)	Lieber-Decarli	100% NRC (mmol/liter)	60% NRC
5	8.53 ± 4.06	9.91 ± 15.05	12.92 ± 18.26
20	3.11 ± 2.28	7.34 ± 3.15	3.52 ± 3.17

Values are the mean ± SD, with five animals per dietary group.

than mice fed the 100% NRC-ETOH or the 60% NRC-ETOH diet (data not shown). Body weights of the 100% NRC-ETOH group were significantly higher than those of the 60% NRC-ETOH group. Only with the 60% NRC diet was a significant difference between ETOH- and pair-feeding the control diet observed.

In mice fed the LD diet, the hepatic vitamin E (α-tocopherol) level was 37.5 ± 4.4 μg/g liver in controls, and mice fed the LD diet containing ethanol had 30.5 ± 3.1 μg/g. This is a 19% decrease. There was 34% less hepatic vitamin A in controls fed the 100% NRC diet and 79% less in controls fed the 60% NRC control diet. In mice fed the 100% NRC diet with added ETOH, there was 81% less vitamin A present in the liver.

Spleen Weight, Spleen Cell Number, and Thymus Weight

Spleen (Fig. 1) and thymus (Fig. 2) weights were not significantly different among mice with free access to the three control diets. ETOH ingestion significantly reduced spleen and thymus weights compared to the control diet ad libitum. With the LD diet, spleen and thymus weights were comparable in ETOH- and pair-fed mice, while with the 100% NRC and 60% NRC diets, ETOH-fed mice had significantly lower spleen and thymus weights than their pair-fed counterparts. Spleen cell number was independent of the dietary composition, but was significantly reduced by ETOH (data not shown).

Cytokine Production

ETOH ingestion significantly increased the capacity of activated splenocytes to produce immunoreactive IL-2 in vitro (Fig. 3). No significant difference among the three diets was measured, however. IFN secretion was strongly influenced by ETOH and dietary composition (Fig. 4). With the LD diet, no significant difference in IFN secretion among the three experimental groups was observed. ETOH ingestion in the 100% NRC and 60% NRC groups significantly enhanced IFN secretion by in vitro activated splenocytes. Chronic ETOH exposure significantly reduced the secretion of immunoreactive TNF by activated splenocytes with the LD and 100% NRC diets (Fig. 5). There was no significant effect of dietary composition on TNF production in vitro.

NK Cell Activity

The lytic activity of NK cells was significantly enhanced in mice fed the LD-ETOH-containing diet, but not in mice fed the other ETOH-containing diets (Fig. 6). The control group for the LD diet showed the lowest NK cell activity, while the NK cell activities of controls fed the 100% NRC and 60% NRC diets were higher (statistically significant difference between LD and 60% NRC diet). Energy restriction by pair-feeding reduced NK cell activity compared to ETOH ingestion in all dietary groups, but only the difference for the LD group was statistically significant.

Phagocytosis

Mice fed control diets ad libitum showed significant differences in their phagocytic capacity. With the LD control diet, the 100% NRC control diet, and the 60% NRC control diet, 53.4 ± 1.5%, 46.9 ± 6.02% ($p < 0.05$), and 37.9 ± 4.17% ($p < 0.05$) of splenocytes, respectively, phagocytized one latex bead (Fig. 7). Splenocytes from 100% NRC control mice incorporated a significantly higher percentage of two latex beads, and splenocytes from 60% NRC control mice incorporated a significantly higher percentage of three or more latex beads than those from LD control mice (Fig. 7). ETOH ingestion and pair-feeding had no effect on the phagocytosis of splenocytes in mice fed the LD diet. ETOH ingestion and pair-feeding significantly increased phagocytic activity in animals fed the 100% NRC diets, while in animals fed the 60% NRC diet, only ETOH ingestion significantly increased phagocytosis.

DISCUSSION

The objective of this study was to investigate the effect of dietary alteration on the immunomodulatory effects of chronic ETOH ingestion. We used as a basic diet the liquid ETOH diet introduced by Lieber-DeCarli.[8,9] Since this diet contains micronutrients in concentrations that far exceed the recommendations for the growth of mice,[14] we devised a liquid diet that more closely resembled these requirements. This diet, the 100% NRC, contained 100% of the recommended concentrations of all essential micronutrients, as defined by the National Research Council.[14] We devised a second diet, the 60% NRC diet, containing 60% of the micronutrient concentrations found in the

Table 3. Food Intake of Mice Fed Various ETOH Diets for 7 Weeks

	L-D*			100% NRC			60% NRC		
	Control	ETOH	PF	Control	ETOH	PF	Control	ETOH	PF
Food intake† (mJ/day/mouse)	57.00 ± 5.06	41.30 ± 3.72‡	41.30 ± 3.72§	56.47 ± 3.89	42.85 ± 6.65‡	42.85 ± 6.65§	55.97 ± 2.30	41.80 ± 6.27‡	41.80 ± 6.27§
ETOH intake (mJ/day/mouse)	0	12.37 ± 1.13	0	0	12.83 ± 2.01	0	0	12.54 ± 1.88	0

* Pair-fed control (PF) diet (matched with consumption of ETOH diet).

† Average daily food intake during the 7-week period.

‡ $p < 0.05$, control vs. ETOH within diets.

§ $p < 0.05$, control vs. pair-feeding within diets.

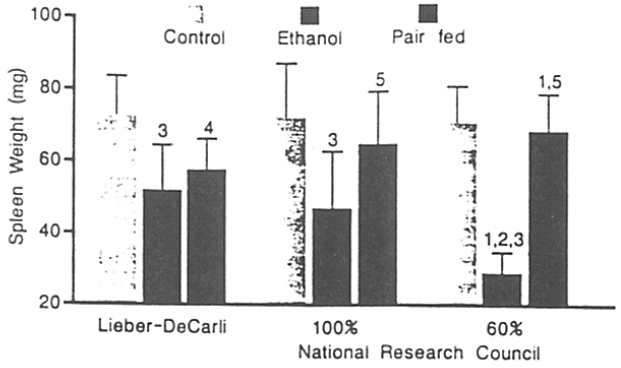

Fig. 1. Spleen weight of mice fed the LD, 100% NRC, and 60% NRC diets and alcohol for 7 weeks. Data are expressed as mean ± SD; numbers indicate significant differences at the level of $p < 0.05$: 1 = 60% NRC vs. LD, 2 = 60% NRC vs. 100% NRC, 3 = control vs. ETOH within diets, 4 = control vs. pair-fed within diets, 5 = ETOH vs. pair-fed within diets.

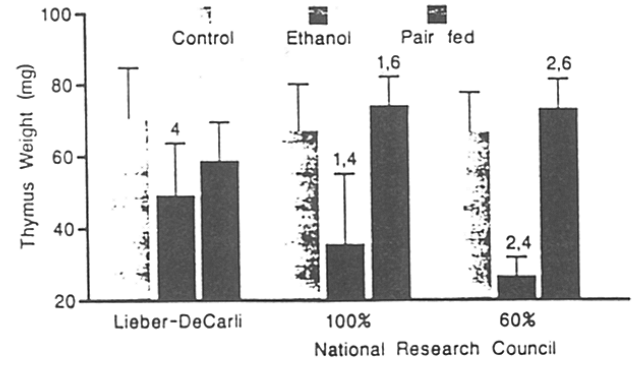

Fig. 2. Thymus weight of mice fed the LD, 100% NRC, and 60% NRC diets and alcohol for 7 weeks. Data are expressed as mean ± SD; numbers indicate significant differences at the level of $p < 0.05$: 1 = LD vs. 100% NRC, 2 = LD vs. 60% NRC, 4 = control vs. ETOH within diets, 6 = ETOH vs. pair-fed within diets.

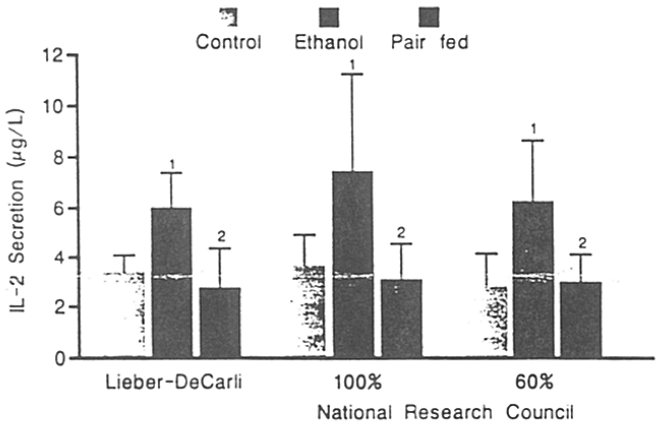

Fig. 3. Interleukin-2 secretion by ConA-activated splenocytes cultured for 48 hr. Mice were fed the LD, 100% NRC, and 60% NRC diets and alcohol for 7 weeks. Data are expressed as mean ± SD; numbers indicate significant differences at the level of $p < 0.05$: 1 = control vs. ETOH within diets, 2 = ETOH vs. pair-fed within diets.

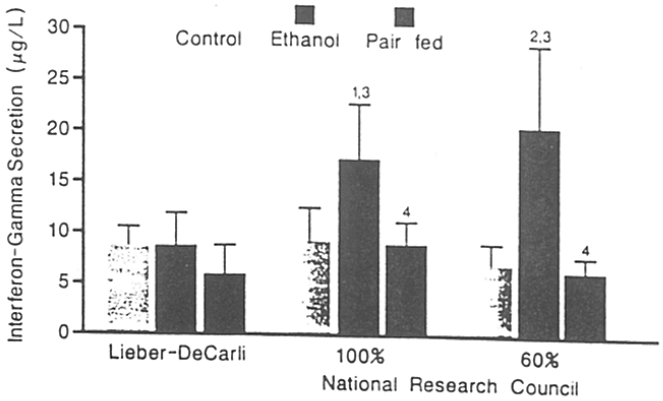

Fig. 4. Interferon-gamma secretion by ConA-activated splenocytes cultured for 48 hr. Mice were fed the LD, 100% NRC, and 60% NRC diets and alcohol for 7 weeks. Data are expressed as mean ± SD; numbers indicate significant differences at the level of $p < 0.05$: 1 = LD vs. 100% NRC, 2 = LD vs. 60% NRC, 3 = control vs. ETOH within diets, 4 = ETOH vs. pair-fed within diets.

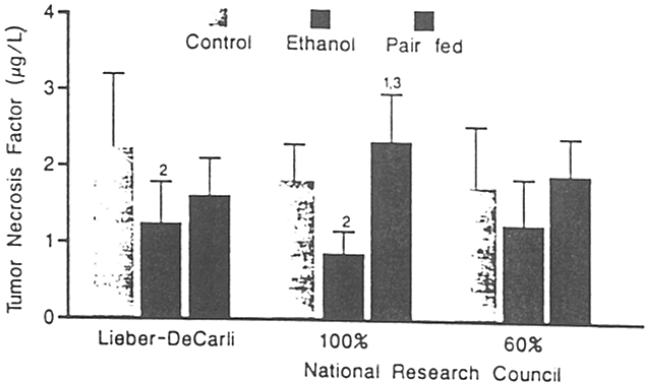

Fig. 5. TNF secretion by LPS-activated splenocytes cultured for 24 hr. Mice were fed the LD, 100% NRC, and 60% NRC diets and alcohol for 7 weeks. Data are expressed as mean ± SD; numbers indicate significant differences at the level of $p < 0.05$; 1 = LD vs. 100% NRC, 2 = control vs. ETOH within diets, 3 = ETOH vs. pair-fed within diets.

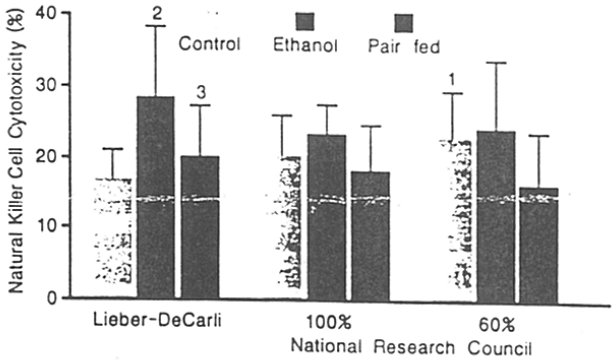

Fig. 6. NK cell activity of splenocytes from mice fed the LD, 100% NRC, and 60% NRC diets and alcohol for 7 weeks. NK cell activity was measured in a 4-hr Cr^{51} release assay against YAC-1 cells and results are presented from experiments with an effector: target ratio of 100:1. Data are expressed as means ± SD; numbers indicate significant differences at the level of $p < 0.05$: 1 = LD vs. 60% NRC, 2 = control vs. ETOH within diets, 3 = ETOH vs. pair-fed within diets.

100% NRC diet, to more closely resemble the micronutrient malnutrition that may occur in chronic alcoholics.[1]

Food intake and body weights of mice with free access to LD, 100% NRC, and 60% NRC control diets were similar, which indicates that our diets were as nutritionally adequate as the LD diet for the growth of mice during the experimental period. The extent to which growth was impaired by incorporating ETOH into the diets depended largely on the nutritional quality of the diets. Mice fed the LD-ETOH diet, which supplied the highest micronutrient concentration of all ETOH diets, showed the highest body weight. With decreasing micronutrient concentrations, the body weights of mice fed ETOH diets declined. Only with the 60% NRC diet, however, did ETOH ingestion combined with an inadequate diet reduce body weights signif-

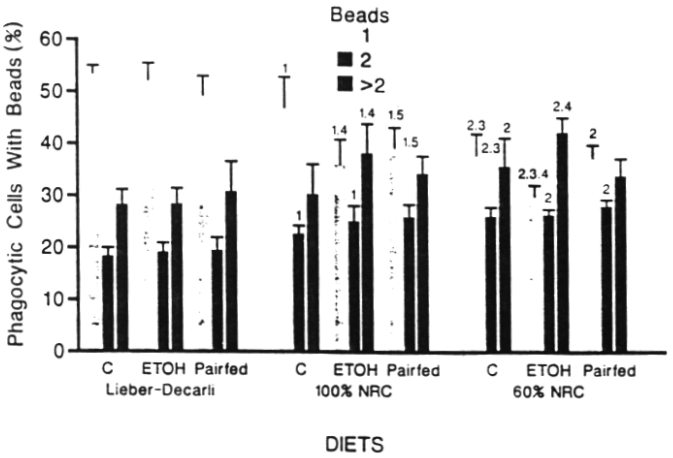

Fig. 7. Phagocytic activity of splenocytes from mice fed the LD, 100% NRC, and 60% NRC diets and alcohol for 7 weeks. The phagocytic activity was determined by flow cytometric quantitation with fluorescent microspheres. Data are expressed as mean ± SD; numbers indicate significant differences at the level of $p < 0.05$: 1 = LD vs. 100% NRC, 2 = LD vs. 60% NRC, 3 = control vs. ETOH within diets, 4 = ETOH vs. pair-fed within diets, 5 = control vs. pair-fed within diets.

icantly beyond the margin that could be explained by the decreased energy intake in ETOH- and pair-fed mice. Other ETOH studies using the LD diet did not observe body weight differences between ETOH- and pair-fed rats.[2,6,9,16]

As with the body weights, the spleen and thymus weights of mice fed the control diets ad libitum showed no difference. In pair-fed mice as well as those exposed to ETOH, the LD diet produced spleen and thymus weights that were lower than those in controls, indicating that the reduced energy intake had caused the low lymphoid organ weights and that ETOH had no effect. In contrast to the LD diet, ETOH as part of the 100% NRC and 60% NRC diets induced significantly lower spleen and thymus weights than those in energy-restricted pair-fed mice. From these data we conclude that the dietary composition of the LD diet in some way protected lymphoid organs from the toxic effects of ETOH, while the lower micronutrient concentrations in the other diets unmasked immunosuppressive ETOH effects. Several investigators using the LD diet,[10,12,13] observed no differences in spleen weights between ETOH and pair-fed animals presumably due to the dietary composition of this diet. Our results further demonstrate that the spleen cell number was significantly reduced by ETOH and was not modified by the diet. The relatively low intake of ETOH with a long exposure time (7 weeks) did not change significantly T-cells subsets in the spleen.[33] However a much higher consumption of ETOH (7% V/V), with dehydration and reduced food intake did alter splenic immune cell architecture.[11]

The capacity of lymphocytes to secrete immunoreactive IL-2 was also highly sensitive to ETOH and was not modified by the diet. Splenocytes from the ETOH-fed animals produced increased levels of immunoreactive IL-2, the first known report of increased IL-2 secretion by ETOH-treated mice.[28] Preliminary data from a recent study using the LD-ETOH diet demonstrated an increased responsiveness of murine splenocytes to various mitogens.[29] ETOH treatment did not affect IFN secretion by splenocytes in mice fed the LD diet, while IFN secretion was enhanced in mice fed the 100% NRC-ETOH or 60% NRC-ETOH diet, demonstrating that IFN secretion was modified by ETOH and diet. As ETOH use lowers levels of hepatic vitamins E and A,[25] which are required for normal immune functions, the dietary composition of the LD diet probably masked any ETOH effect. According to a recent review, no other studies have been published reporting an effect of ETOH in vivo on IFN secretion.[28] The secretion of immunoreactive TNF was suppressed by ETOH with the LD and 100% NRC diets. In earlier studies we observed a similar reduction in the secretion of immunoreactive TNF by rat Kupffer cells after chronic ETOH treatment[30] using the LD diet. Mice fed these regimens and alcohol had essentially no change in spleen lymphocyte subsets.[31] Thus, changes in cytokine production or NK activity were not likely due to major changes in lymphocyte subsets. If alcohol damaged intestinal integrity there could be more LPS exposure to cells, which would stimulate cytokine production by activation of cells.

NK cell activity was also affected by the nutritional quality of the diet. Decreasing the nutritional quality of the control diets consumed ad libitum to the recommended level for normal growth resulted in increased NK cell activity. NK cell activity was further enhanced in mice fed the LD-ETOH diet and was not moderated in mice fed the 100% NRC-ETOH or 60% NRC-ETOH diet. An earlier study, in which mice were fed diets with micronutrient levels comparable to those of the LD diet, also reported enhanced NK cell activity with chronic ETOH treatment,[30] while in rats no differences between ETOH and controls were observed.[13] From our data we conclude that energy restriction by pair-feeding reduces NK cell activity. This confirms the findings of another study, which similarly reported that pair-feeding lowered NK cell activity.[32] This indicates that either the energy restriction itself or neuroendocrinological mechanisms related to such stress may have caused the reduction in NK cell activity.

The phagocytic capacity of splenocytes was significantly affected by the composition of the diets. Feeding the LD control diet resulted in low phagocytic activity, while feeding the 100% NRC or 60% NRC control diet increased the phagocytic activity. Mice fed the diet with the lowest micronutrient concentration expressed the highest phagocytic activity. The LD-ETOH diet had no effect on the phagocytic activity, which conflicts with earlier studies, where we found a suppression of the phagocytic activity in rat alveolar and murine peritoneal macrophages.[13,33] The 100% NRC diet revealed an increase in the phagocytic activity by energy restriction. In addition to the stimulation of phagocytic activity by energy restriction in the

100% NRC group, the 60% NRC group demonstrated a further stimulation of phagocytosis by exposure to ETOH. Such changes could be due to stimulation by increased exposure to bacterial products from ETOH or malnutrition damage to defenses of the liver and intestine.[37]

These findings clearly suggest that ETOH-containing diets stimulate or suppress the immune system by at least three mechanisms. Some of these parameters are directly influenced by ETOH without any nutritional modulation. To this group belong spleen cell number and the capacity of splenocytes to secrete immunoreactive IL-2 and TNF. A second group consists of immunological parameters by which the effects of ETOH are further modulated by the nutritional quality of the diet. Parameters belonging to this second group are body, spleen, and thymus weights, IFN secretion, NK cell activity, and phagocytic activity. The third group consists of immune mechanisms that can be directly affected by the nutritional quality alone, as we have demonstrated for NK cell and phagocytic activity.

The intense interaction between chronic ETOH ingestion and nutritional status on one side and between nutritional status and immunocompetence on the other creates many ways in which nutrition can affect ETOH-induced immunomodulation. It is apparent from animal and human studies that in the absence of ETOH consumption, excesses or deficiencies of various nutrients can directly affect immune functions.[36] Vitamin A, particularly, modulates immune responses in various ways. Vitamin A deficiency in rats decreased NK cell activity and IFNα/β production,[17] and impaired the antibody response.[19] High concentrations of vitamin A induced in murine peritoneal macrophages resulted in increased phagocytic and tumoricidal activity and IL-1 production.[18] The LD diet contains extremely high concentrations of vitamin A, which can stimulate these immune responses and counteract the vitamin A-depleting effect of chronic ETOH ingestion. Additionally, high dietary intakes of vitamin E (100–2500 mg/kg diet; LD diet contained 120 mg/kg diet) stimulated splenic NK cell and phagocytic activity of alveolar macrophages.[34] The high antioxidant concentration in the LD diet could protect lymphoid cells from damage by oxygen radicals generated during metabolism of ETOH. In addition to the effects of the micronutrients, the quality of the fatty acids used in the various diets might have modified immune responses and the immunomodulating effects of ETOH.[35]

Clearly, diets used in experimental studies of ETOH-induced immunomodulation should be planned and controlled carefully, as the dietary composition itself can affect the immune response and further modulate the immunological effects of ETOH. The use of well established ETOH diets may not be appropriate for investigation of the immunomodulating effects of chronic ETOH ingestion and may confound the data generated in any study of this type. Clearly, diets with megadoses of vitamins do not duplicate those of human alcoholics.[2] The increased mortality in mice fed the NRC alcohol diets may be particularly useful to the model loss of disease resistance seen in human alcoholics. The amount of alcohol and exposure time can be adjusted to prevent mortality. Further animal studies are necessary with diets that are as close as possible to the actual nutritional requirements of the animals and include an adequate safety margin and which more closely resemble the nutritional condition in chronic alcoholics. Finally, the nutritional supplementation of alcoholic beverages or of alcohol abusers may help to compensate for ETOH-induced malnutrition, but this will not prevent the direct immunomodulatory effects of ETOH.

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Alterations in Mouse Peyer's Patch Lymphocyte Phenotype After Ethanol Consumption

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LOPEZ, M. C., B. WATZL, L. L. COLOMBO, AND R. R. WATSON. *Alterations in mouse Peyer's patch lymphocyte phenotype after ethanol consumption.* ALCOHOL **14**(2) 107–110, 1997.—The objective of this study was to determine if chronic ethanol consumption could modify cell populations in the Peyer's patches (PP), which could favor pathogenic or opportunistic infections in mice, as seen in chronic alcohol addicts. Young C57BL/6 mice receiving the Lieber-DeCarli diet (36% of calories as ethanol) for 5 weeks presented a significant decrease in the total number of cells in the PP. Mature FVB mice receiving the Lieber-DeCarli diet for 19 weeks presented a highly significant decrease in the total number of cells and in the absolute number of T and B cells in the PP. Young C57BL/6 mice receiving the 100%NRC (30% ethanol) or the 60% NRC (30% ethanol) diets for 7 weeks presented alterations in the T and B cell phenotype comparable with the alterations observed in mice receiving the Lieber-DeCarli diet for 19 weeks. As less alcohol for a shorter time caused similar changes to those seen with a highly micronutrient enriched diet with more alcohol for a longer consumption period, micronutrient supplementation may overcome some immune damage found in animal models of alcoholism. Our data indicated that ethanol administration altered the mucosal immune system at the level of the PP, the site for antigen presention and induction of a mucosal immune response. © 1997 Elsevier Science Inc.

Ethanol Lieber-DeCarli diet NRC diet Peyer's patches Mucosal immunology Lymphocyte phenotype

ALCOHOL abusers have more frequent bacterial infections including pneumonia and higher infection-associated mortality than nonalcoholics (9). The severe effects of orally administered ethanol on the immune system (2,13,15) particularly in spleen (14) and thymus (12) cell populations have been shown using different experimental designs. The loss of lymphocyte numbers in the spleen and thymus (12) as well as the abnormal functionality of the remaining lymphocytes (1,3,11) have been demonstrated using high concentration short-term ethanol exposure protocols (7% v/v for 7–10 days in liquid diets).

Most alcoholics imbibe continuously or with a regular periodicity using the same or increasing doses of alcohol over a prolonged period of time. Chronic alcohol addicts usually suffer undernutrition associated with changes in their lifestyles (15). Therefore, we decided to use three different diets that could represent an overnourished, a well-nourished and a partially undernourished population, based on the guidelines of the National Research Council (10,16). The changes induced by ethanol consumption on the mucosal immune system using longer exposures—5, 7, and 19 weeks—were evaluated because we were interested in the immune alterations associated with chronic alcoholism. Different exposure times were necessary to establish the toxic additive effect of ethanol on the immune system.

The objective of this study was to determine if ethanol could alter the mucosal immune system at the level of Peyer's patches (PP) and mesenteric lymph nodes (MLN). Moreover, we assessed whether the length of ethanol exposure and the nutritional adequacy of the diet administered were co-factors of the ethanol-induced damaged.

METHOD

Animals

Although young mice with somewhat immature immune systems are routinely used in alcohol model studies, immunologically mice may better model human alcoholics. Their immune systems may be more resistant to damage. Male C57BL/6 and female FVB mice, 4 weeks old, were obtained from the National Cancer Institute (Frederick, MD). Female C57BL/6 mice were obtained from Charles River Laboratories, Inc. (Wilmington, DE). Mice were kept in the animal facility at the Arizona Health Science Center until they were assigned to the differ-

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TABLE 1

EFFECT OF LIEBER-DeCARLI DIET (36% ETHANOL) FOR 5 WEEKS ON THE ABSOLUTE NUMBER OF T AND B CELLS IN THE PEYER'S PATCHES OF C57BL/6 MICE

Diet	Total No. of Cells (x10^6)	Absolute Number of T and B Cells (x10^4)			
		CD4$^+$	CD8$^+$	sIgA$^+$	sIgM$^+$
Control (8)	2.9 ± 0.7	5.0 ± 3.1	4.1 ± 1.6	11.7 ± 2.1	12.7 ± 1.2
ETOH (8)	1.9 ± 0.7*	3.9 ± 3.8	4.5 ± 4.6	8.3 ± 4.9	8.0 ± 4.4

Results are expressed as mean ± standard deviation. ( ) = number of mice.
Significantly different from the control group: *$p < 0.05$.

ent experimental groups. Animals were cared for as required by the University of Arizona College of Medicine Committee on Animal Research.

Diets

Male C57BL/6, 5 weeks old, and female FVB, 7 and 20 weeks old, were fed the Lieber-DeCarli liquid diets (Dyets, Bethlehem, PA) without ethanol (ETOH) or with 36% of the energy derived by ETOH, for 5 and 19 weeks, respectively (4). The young mice, recently weaned, still have developing immune systems, while immunology mature mice (20 weeks old) are at optimum immune function. Female C57BL/6 mice, 5 weeks old, received the liquid 100% National Research Council (NRC) (10) diet with 30% of the energy derived by ETOH and the liquid 60% NRC diet (Dyets, Bethlehem, PA) with 30% of the energy derived by ETOH for 7 weeks as described previously (16–18). Both diets were given without ETOH as an isocaloric control diet.

Lymphocyte Subpopulations Measurement

Peyer's patches (PP) and mesenteric lymph nodes (MLN) were collected after sacrifice under ether anesthesia and mononuclear cells were obtained by gently teasing the tissues through stainless steel wire mesh screens in RPMI-1640 medium supplemented with 10% fetal calf serum. Cell suspensions were washed twice with cold medium, viability was determined by trypan blue exclusion, and the cell suspensions were adjusted to give the desired viable cell concentrations ($2–3 \times 10^5$ cells/0.1 ml/tube) for lymphocyte surface marker determinations. Then, T-cell subpopulations were quantified with indirect immunofluorescence using monoclonal antibodies (Seralab, Accurate Chemicals, Westbury, NY) specific for T-helper cell (rat IgG2b antimouse CD4 antigen) T-suppressor cells (rat IgG2b antimouse CD8 antigen). Fluorescein isothiocyanate labeled goat antirat immunoglobulin (heavy and light chain specific) (Southern Biotechnology Associates, Birmingham, AL) was used as a secondary antibody. B cells that express IgA or IgM were analyzed by indirect immunofluorescent staining by using goat antimouse IgA (α-chain specific) or goat antimouse IgM (μ-chain specific) and a FITC-conjugated rabbit antigoat as a secondary antibody (Cappel Division of Organon Teknika, Durham, NC). The staining procedure was performed as described previously (5,6). The stained cells were fixed in 0.3 ml of 2% paraformaldehyde and were stored at 4–8°C until they were analyzed by a FACScan flow cytometer. The percentage of positive cells was obtained by using the Consort 30 Program provided by Becton Dickinson. Five thousand cells were counted. The absolute number of cells labeled with the specific monoclonal antibody was calculated by multiplying the total number of cells by the percentage of positive cells.

Statistical Analysis

Data were analyzed using the two-tail Student's *t*-test.

RESULTS

Effect of Lieber-DeCarli Diet (36% ETOH) for 5 Weeks on PP and MLN Lymphocytes of Young C57BL/6 Mice

Routinely, others as well as ourselves have used 36% of calories as ethanol with this diet to get immunosuppression (2,12,14). Short-term administration of a diet designed to have an excess of micronutrients was supplemented provides 36% of the energy by ETOH to young male mice. It provoked a significant decrease in the total number of cells in the PP (Table 1).

Nevertheless, the absolute number of cells bearing the specific surface markers evaluated (CD4, CD8, sIgA, and sIgM) was not modified. No changes were detected either in the total

TABLE 2

EFFECT OF LIEBER-DeCARLI DIET (36% ETHANOL) FOR 5 WEEKS ON THE ABSOLUTE NUMBER OF T AND B CELLS IN THE MESENTERIC LYMPH NODES OF C57BL/6 MICE

Diet	Total No. of Cells (x10^{-6})	Absolute Number of T and B Cells (x10^{-6})			
		CD4$^+$	CD8$^+$	sIgA$^+$	sIgM$^+$
Control (7)	4.6 ± 0.7	2.4 ± 0.3	1.8 ± 0.3	0.9 ± 0.2	1.1 ± 0.1
ETOH (7)	5.9 ± 2.5	2.9 ± 1.5	2.5 ± 1.4	1.4 ± 1.0	1.3 ± 0.5

No significant differences were observed between the control and experimental groups.

TABLE 3

EFFECT OF DIETS WITH DIFFERENT NUTRITIONAL ADEQUACY AND DIFFERENT LEVLES OF ETHANOL FOR 7 WEEKS ON THE ABSOLUTE NUMBER OF T AND B CELLS IN THE PEYER'S PATCHES OF C57BL/6 MICE

Diet	Total No. of Cells (x10^{-6})	Absolute Number of T and B Cells (x10^{-5})			
		CD4$^+$	CD8$^+$	sIgA$^+$	sIgM$^+$
100:C (4)	3.9 ± 0.1	14.6 ± 1.3	6.1 ± 0.1	19.6 ± 2.8	23.8 ± 3.3
100:30 (6)	2.4 ± 0.4*	8.0 ± 0.9*	6.5 ± 0.6	10.0 ± 1.7*	12.8 ± 1.3*
60:C (4)	6.6 ± 0.3	21.0 ± 1.0	23.0 ± 4.5	36.1 ± 6.4	46.4 ± 3.5
60:30 (6)	1.9 ± 1.3*	11.0 ± 5.0*	11.0 ± 4.8*	9.4 ± 6.6*	12.6 ± 7.0*

100:C = 100% NRC control; 100:30 = 100% NRC + 30% ethanol; 60:C = 60% NRC control; 60:30 = 60% NRC + 30% ethanol. Significantly different from the respective control group: *$p < 0.005$.

number of cells or in the absolute number of T and B cells studied in the MLN of these mice (Table 2).

Effect of 100% and 60% NRC Diets Containing 30% ETOH for 7 Weeks on PP Lymphocytes of Young C57BL/6 Mice

To observe the effects of reduction of nutrients the level of dietary was reduced from the maximum tolerated (36%) to 30% of calories as done previously (17). Short-term administration of a diet designed to have an adequate amount of micronutrients for growth was supplemented to provide 30% of the energy by ETOH to young female mice provoked a significant decrease in the total number of cells and in the absolute number of CD4$^+$, sIgA$^+$, and sIgM$^+$ cells in the PP (Table 3).

A liquid diet designed to provide 60% of the NRC requirements and providing 30% of the energy by ETOH when administered to young female mice provoked a significant decrease in the absolute number of T and B cells in the PP (Table 3).

Effect of Lieber-DeCarli Diet (36% ETOH) for 19 Weeks on PP and MLN Lymphocytes of Mature FVB Mice

Long-term administration of a nutritionally enriched diet providing 36% of the energy by ETOH induced a significant decrease in the absolute number of CD4$^+$, CD8$^+$, sIgA$^+$, and sIgM$^+$ cells in the PP of FVB female mice (Table 4). However, no changes were observed in the MLN of these mice (data not shown).

DISCUSSION

The objective of this study was to determine whether chronic ethanol consumption could modify cell populations in the mucosal immune system thereby favoring pathogen or opportunistic infections as seen in chronic alcohol addicts (9,13). The higher incidence of bronchial and intestinal infections among chronic alcoholics, as compared to nonalcoholic individuals, motivated our study. In previous articles we evaluated changes at the level of the intestinal lamina propria, the effector site of the mucosal-associated immune system (17,8), whereas in this article we focused on the PP, the inductive site for a mucosal immune response.

In the present report, we used mice that at the beginning of the study were young or mature trying to detect if the age at which they began consumption of ethanol could be a cofactor for immune changes. We used male mice for short-term ethanol exposure studies. For longer exposure times we preferred to use female mice because males showed more tendency to fight with each other, adding stress as an extra nondesirable cofactor to our study.

Young C57BL/6 male mice receiving the nutritionally enriched Lieber-DeCarli diet plus ethanol for 5 weeks showed a decrease in the total number of cells in the PP with no changes in the absolute number of T and B cells (Table 1). In previous experiments, using young female mice fed the Lieber-DeCarli diet supplemented with ethanol for 11 weeks, we were not able to detect changes in T or B cell populations in the intestinal lamina propria (ILP) (8). However, when the NRC diets supplemented with ethanol were used, we demonstrated an in-

TABLE 4

EFFECT OF LIEBER-DECARLI DIET (36% ETHANOL) FOR 19 WEEKS ON THE ABSOLUTE NUMBER OF T AND B CELLS IN THE PEYER'S PATCHES OF FVB MICE

Diet	Total No. of Cells (x10^{-6})	Absolute Number of T and B Cells (x10^{-5})			
		CD4$^+$	CD8$^+$	sIgA$^+$	sIgM$^+$
Control (8)	7.4 ± 1.8	27.0 ± 8.0	11.1 ± 5.0	17.8 ± 12.3	17.3 ± 11.1
ETOH (6)	1.8 ± 1.0*	6.3 ± 0.5*	1.9 ± 0.7*	3.4 ± 0.1‡	3.7 ± 0.5‡

Significantly different from the control group:
*$p < 0.01$.
†$p < 0.05$
‡$p < 0.02$.

crease in the number of CD8 cells in the ILP of young mice (7). Moreover, when mature mice were evaluated, an increase in the number of CD4 and CD8 cells in the ILP of those mice that received the 100% NRC diet with 30% of the energy derived by ETOH was observed (7). In this study, young C57BL/6 female mice receiving the 100%NRC or the 60% NRC ETOH diets for 7 weeks showed alterations in the T and B cell phenotype that included a significant decrease in the absolute number of $CD4^+$ T cells and B cells in the PP. These results were comparable with the alterations observed in mice that received the Lieber-DeCarli diet for 19 weeks (Table 3).

Mature FVB female mice receiving the Lieber-DeCarli diet for 19 weeks presented a highly significant decrease in the total number of cells and in the absolute number of T ($CD4^+$, $CD8^+$) and B ($sIgA^-$, $sIgM^+$) cells in the PP (Table 4). None of the experimental groups showed changes in the MLN T or B cell populations. The Lieber-DeCarli diet contains micronutrients in concentrations much higher than the recommendations for mouse growth (10) for that reason it was considered a nutritionally enriched diet. As the Lieber-DeCarli diet contains high levels of immunomodulatory vitamins (16), it could mask the immunosuppressive effects of ethanol and thereby it could explain why a prolonged exposure was necessary to detect the same phenotypical alterations observed after 7 weeks of intake in the NRC diet-fed mice. On the other hand, the 100% NRC diet fulfills the mouse requirements for growth, whereas the 60% NRC diet provides only 60% of the vitamin and mineral requirements (17). Therefore, the extent of nutritional supplementation above the recommended intake can be important to facilitate or prevent ethanol-induced alterations.

In conclusion, our data showed that ethanol administration impaired the mucosal immune system at the level of the Peyer's patches, that is, the site for antigen presention and, therefore, for induction of a mucosal immune response. Moreover, our data suggest that the effect of ethanol may be delayed or masked if a nutritionally enriched diet was simultaneously consumed.

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ORIGINAL CONTRIBUTION

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Acute intake of moderate amounts of red wine or alcohol has no effect on the immune system of healthy men

Summary *Background* A recent prospective cohort study revealed that moderate wine consumption but not consumption of other alcoholic beverages is associated with a decreased risk of common cold. In contrast, wine constituents such as ethanol and polyphenols are known to suppress immunity. *Aim of the study* We investigated whether acute intake of a moderate amount of alcohol modulates immune functions in healthy men and whether polyphenols in red wine with antioxidative and immunomodulatory potential induce changes in immune functions that differ from those induced by the consumption of the 12% ethanol. *Methods* Six healthy males with moderate alcohol consumption patterns randomly consumed a single dose of 500 ml of red wine (12% ethanol), a 12% ethanol dilution, dealcoholized red wine, and red grape juice, respectively. The following immune functions were measured before beverage consumption and 1, 3, and 24 h later: phagocytic activity and intensity of neutrophils and monocytes, production of tumor necrosis factor-alpha, interleukin-2, and interleukin-4, lymphocyte proliferation, and lytic activity of natural killer cells. *Results* Acute consumption of a moderate amount of red wine and of a 12% ethanol solution had no effect on immune functions in men. Acute consumption of polyphenol-rich beverages (dealcoholized red wine and red grape juice) also did not affect immunity. *Conclusions* This study clearly shows that moderate consumption of alcohol at doses which inversely correlate with cardiovascular disease risk has no short-term effect on human immune cell functions. Acute intake of polyphenol-rich beverages such as red grape juice and dealcoholized red wine also does not affect immunity.

Key words red wine – red grape juice – ethanol – anthocyanins – immune system

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Introduction

In epidemiological studies moderate alcohol consumption has been shown to protect people from coronary heart disease (Renaud et al. 1998, Rimm et al. 1999, Gaziano et al. 1999, Gronbæk et al. 2000). Furthermore, moderate intake of alcohol (up to three drinks) in nonsmokers intentionally exposed to rhinoviruses was associated with a decreased risk of the common cold (Cohen et al. 1993) suggesting that alcohol may stimulate the immune response against this type of virus. In a recent prospective cohort study, total alcohol intake, beer and spirits consumption were not related to the occurrence of common cold, whereas moderate consumption of wine was inversely associated with the risk of common cold. The association was stronger for red wine when compared with white wine (Takkouche et al. 2002) indicating that specific polyphenols in red wine contribute to the observed reduction in common cold risk.

In contrast, consuming more than three drinks of alcohol per day is associated with an increased all-cause mortality (Bofetta and Garfinkel 1990). Chronic ethanol abuse is known to result in specific defects in innate and acquired immunity and may increase host susceptibility to infections (Watzl and Watson 1992, Nelson and

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Kolls 2002). Recent experimental evidence suggests that acute, moderate alcohol consumption can also affect the immune system. Pharmacological doses of ethanol *in vitro* significantly suppress the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) in human peripheral blood mononuclear cells (PBMC) (Watzl and Watson 1993, Szabo et al. 1996). Moreover, ethanol exposure of polymorphonuclear neutrophilic granulocytes (PNG) and monocytes *in vitro* and *in vivo* inhibits phagocytosis (Stoltz et al. 1999) and decreases their capacity to produce reactive oxygen species (Patel et al. 1996, Szabo 1999). However, relatively little is known of the effects of acute alcohol consumption on the immune system of humans (Szabo 1999).

Alcohol consumption and its subsequent metabolism in the liver generates reactive oxygen species, which interfere with various immune cell functions. Phenolic antioxidants in red wine may scavenge reactive oxygen species and thereby protect the host against impairment of immune cell functions due to ethanol intake. Based on the French wine consumption of 180 ml/d, the daily intake of polyphenols from wine can be estimated to be as high as 500 mg/person (Teissedre and Landrault 2000). Recently, in an animal study it was shown that ethanol intake in the form of red wine produced no changes in blood lymphocyte and NK cell numbers, while a similar intake of ethanol alone reduced the percentage of these cells (Percival and Sims 2000). This suggests that polyphenols in red wine may prevent toxic effects of high ethanol doses. Immune cell functions, however, were not measured in this study. Red wine contains a variety of polyphenols with strong *in vitro* as well as *in vivo* antioxidant (Wang et al. 1997, Casalini et al. 1999, Lodovici et al. 2001) and immunomodulatory effects (Middleton et al. 2000). Despite this, the immunological effects of flavonoids in humans have not been studied yet. Most polyphenol studies were performed in *in vitro* systems demonstrating that flavonoids suppress a variety of immune functions including lymphocyte proliferation, lytic activity of NK cells, and cytokine secretion (Middleton et al. 2000).

The objective of this study was first to investigate 1) whether the acute consumption of a moderate amount of ethanol modulates immune functions in healthy men during a period of 24 h, and 2) whether polyphenols in red wine with their antioxidative and immunomodulatory potential induce changes in immune functions that differ from those induced by the consumption of a 12% ethanol beverage.

Subjects and methods

Subjects and study design

Six non-smoking men with moderate alcohol consumption patterns and with normal body weight were recruited for the study. All subjects were in good medical health as determined by a screening history and medical examination. None were taking vitamin supplements or medications two months before or during the study. The study was approved by the Medical Ethical Committee of the Landesärztekammer Baden-Württemberg and all participants gave their consent in writing.

The study was performed under strictly-controlled conditions at the human nutrition unit of the Federal Research Centre for Nutrition within a period of 4 weeks. During the study period, subjects adhered to their usual diets, but were instructed to avoid food products rich in anthocyanins or polyphenols (Bub et al. 2001). Each volunteer had 4 experimental treatments: after an overnight fast, subjects consumed 500 mL of red wine (RW; 12% ethanol v/v), dealcoholized red wine (DRW), red grape juice (RGJ), or ethanol (ETOH; 12% ethanol v/v), with a wash-out period of one week between each experimental day. The subjects were randomly assigned to one of the 4 groups; each subject received the beverages in a different order. With the beverages subjects ate 2 white rolls (150 g). Two hundred min after breakfast, subjects consumed a standardized meal, and further meals until the end of the 24 h-study period were provided to all study subjects.

RW (variety Lemberger) and RGJ (variety Lemberger) grown in the same vineyard were obtained from the State Winery Weinsberg (Baden-Württemberg, Germany). Dealcoholization of the RW was achieved by a vacuum rectification process (Center for dealcoholization EAZ Petershans GmbH, Waiblingen, Germany). The anthocyanin (mg/L) and catechin (mg/L) contents were 171.1 and 63.4 (RW), 144.8 and 47.0 (DRW), and 338.6 and 64.3 (RGJ), respectively. The 12% ethanol beverage was prepared from vodka.

Collection and preparation of blood samples

Fasting venous blood samples were collected before, 1, 3, and 24 h after beverage consumption. Blood was drawn from an antecubital vein into prechilled tubes containing Li-heparin (Monovette-Sarstedt, Nümbrecht, Germany) and immediately placed on ice in the dark. Plasma was collected after centrifugation at 1500 x g for 10 min at 4 °C. Tubes without anticoagulant (Serum-Monovette-Sarstedt, Nümbrecht, Germany) were used for serum collection. Blood was allowed to clot at RT for 30 min, then centrifuged at 1500 x g for 10 min at RT. The serum was stored at –80 °C until analysis.

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Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma, Deisenhofen, Germany) and resuspended in complete RPMI-1640 culture medium (Life Sciences, Eggenstein-Leopoldshafen, Germany), containing 5% (v/v) heat-inactivated FBS (Life Sciences), L-glutamine (2 mmol/L), penicillin (100,000 U/L) and streptomycin (100 mg/L).

Blood ethanol concentration. Blood ethanol concentrations were measured using a commercial kit (Hoffmann-LaRoche, Mannheim, Germany).

Lymphocyte proliferation. PBMC at 1 x 10^9 cells/L in medium containing 10% of FBS were stimulated by the T cell mitogen concanavalin A (5 mg/L, ConA, Sigma, Deisenhofen, Germany) for 120 h at 37 °C. Proliferation was measured using the pyrimidine analogue 5-bromodeoxyuridine, which was quantified in PBMC by a cellular enzyme immunoassay as described earlier (Watzl et al. 2000).

Quantification of cytokine secretion. PBMC at 1 x 10^9 cells/L were cultured in medium containing 10% of FBS and stimulated by 5 mg/L ConA for 24 h at 37 °C (IL-2, IL-4) or by 1 µg/L LPS (TNFα). Cell-free supernatants were collected and stored at –80 °C until analysis. TNFα, IL-2 and IL-4 were measured by sandwich-ELISAs as described previously (Watzl et al. 2000).

Lytic activity of NK cells. Lytic activity of NK cells against K562 target cells (effector:target ratios 50:1, 25:1, 12.5:1) was measured with the flow cytometric method (Watzl et al. 2000).

Phagocytic activity and intensity. Assessment of phagocytic activity (percentage of phagocytic-active cells) and phagocytic intensity (number of phagocytized *E. coli* expressed as mean fluorescence) was based on a recently described flow cytometric method (O'Gorman 2002). Briefly, to measure phagocytic capacity 10 µL isothiocyanate-labeled *E. coli* and 100 µL whole blood were mixed and incubated at 37 °C for 10 min. Reaction was stopped by adding 100 µL ice-cold quenching solution (10% Trypan in PBS). After washing, whole blood cells were fixed with 100 µL FACS-lysing solution (diluted 1:10, BD, Heidelberg, Germany). DNA was stained with 300 µL propidium iodide solution (33.3 mg/L, dissolved in PBS). A FACSCalibur flow cytometer (BD, Heidelberg) was used to measure the level of phagocytic activity and intensity in neutrophils and monocytes.

Statistical analyses

Results are reported as means ± standard error of the mean (SE). Changes between the baseline (0 h) and the following time-points among treatment groups was tested for significance by repeated-measures ANOVA with Fischer's test for comparison of individual means when appropriate. All statistical calculations were performed with the StatView program (SAS Institute 1998, Cary, NC, USA).

Results

The anthropometric data of the study subjects are summarized in Table 1. All participants tolerated the intervention with the 4 beverages very well and completed the study. Maximum blood ethanol concentrations in subjects consuming RW were 15.9 mM and 15.0 mM in subjects consuming ETOH.

The capacity of activated PBMC to produce the cytokines TNFα, IL-2, and IL-4 did not differ at baseline between subjects in the 4 groups. During the 24 h period following the consumption of the beverages, no specific effects were observed (Fig. 1). For TNFα and for IL-4, however, with all beverages a significant time-effect was seen ($p < 0.0001$ and $p = 0.029$, respectively). Phagocytic activity and intensity of neutrophils and monocytes ex vivo were not different at baseline and were not significantly affected by the consumption of the different beverages (Table 2). A significant time effect ($p < 0.0001$) was observed for phagocytic activity of neutrophils independent of the type of beverage consumed. Lymphocyte responsiveness to mitogen activation was also not affected by the beverages during the 24 h period (Table 3). The lytic activity of NK cells also showed for all beverages a significant time-effect ($p < 0.0001$), while there were no effects of the treatment on NK cell function (Table 3; data are only shown for the effector:target ratio of 25:1).

Discussion

Regular consumption of moderate amounts of wine is associated with a reduced risk of cardiovascular disease (Renaud et al. 1998, Rimm et al. 1999, Gaziano et al. 1999, Gronbæk et al. 2000) and of common cold (Takkouche et al. 2002). In the present study the immunomodulatory

Table 1 Anthropometric data of the study subjects (n = 6, mean ± SE)

Age (yr)	31 ± 1.6
Height (cm)	182 ± 2.9
Body mass (kg)	78 ± 4.9
BMI (kg/m^2)	24 ± 1.6

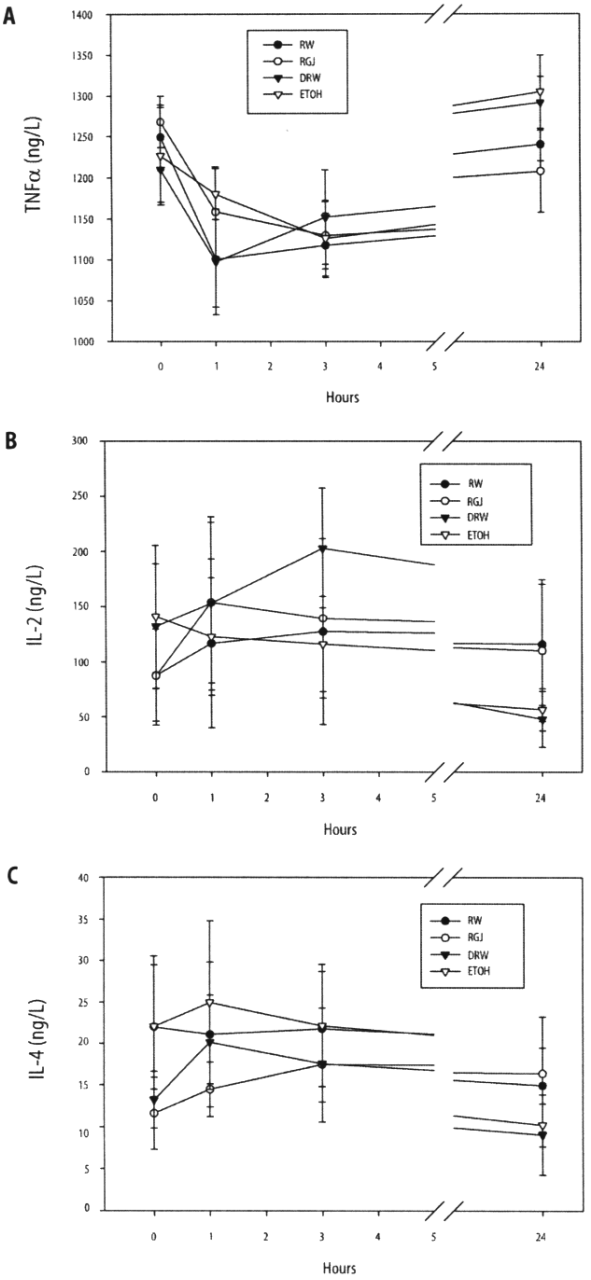

Fig. 1 Production of TNFα, IL-2, and IL-4 by activated peripheral blood mononuclear cells isolated from subjects consuming a single dose of 500 mL red wine (RW), dealcoholized red wine (DRW), red grape juice (RGJ) or a 12 % ethanol control beverage (ETOH) at time point 0, and 1, 3, and 24 h post intakes (n = 6, mean ± SE). **A** TNFα, **B** IL-2, **C** IL-4. A significant time-effect for TNFα and IL-4 was observed by ANOVA independent of the type of beverage consumed (TNFα, $p < 0.0001$; IL-4, $p = 0.03$)

Table 2 Phagocytic activity (percentage of phagocytic-active cells) and phagocytic intensity (mean fluorescence per phagocyte) of peripheral blood mononuclear cells isolated from subjects consuming a single dose of 500 mL red wine (RW), dealcoholized red wine (DRW), red grape juice (RGJ) or a 12 % ethanol control beverage (ETOH) at time point 0, and 1, 3, and 24 h post intakes (n = 6, mean ± SE)

	Time (h)			
	0	1	3	24
Phagocytic activity – neutrophils (%)[1]				
RW	40±4	41±4	54±5	53±5
DRW	35±5	37±6	42±4	47±3
RGJ	42±3	41±3	49±4	50±3
ETOH	41±3	41±4	49±5	50±2
Phagocytic intensity – neutrophils (mean fluorescence per neutrophil)				
RW	64±5	66±4	67±6	66±4
DRW	70±7	76±8	71±5	70±4
RGJ	63±3	66±6	67±5	67±2
ETOH	73±6	72±5	70±5	75±5
Phagocytic activity – monocytes (%)				
RW	26±3	27±3	27±4	28±3
DRW	26±6	26±6	26±5	24±3
RGJ	24±5	24±4	25±5	29±2
ETOH	23±3	20±3	22±4	25±4
Phagocytic intensity – monocytes (mean fluorescence per monocyte)				
RW	64±4	65±6	65±7	67±4
DRW	66±4	67±5	71±5	72±4
RGJ	65±6	70±6	63±4	63±4
ETOH	69±3	67±5	65±4	76±4

[1] A significant time-effect was observed by ANOVA independent of the type of beverage consumed ($p < 0.0001$)

Table 3 Proliferation and NK cell activity of peripheral blood mononuclear cells isolated from subjects consuming a single dose of 500 mL red wine (RW), dealcoholized red wine (DRW), red grape juice (RGJ) or a 12 % ethanol control beverage (ETOH) at time point 0, and 1, 3, and 24 h post intakes (n = 6, mean ± SE)

	Time (h)			
	0	1	3	24
Lymphocyte proliferation (A_{450}–A_{650})				
RW	0.96±0.09	0.90±0.09	1.12±0.11	0.91±0.23
DRW	1.03±0.14	0.86±0.13	0.91±0.15	0.90±0.17
RGJ	0.78±0.22	0.73±0.18	0.95±0.21	1.06±0.15
ETOH	1.07±0.13	1.04±0.07	1.17±0.16	1.09±0.10
NK cell activity (% lysed K562 target cells)[1,2]				
RW	61.5±2.3	55.9±2.5	56.8±3.6	61.1±2.0
DRW	62.7±3.4	46.8±7.5	50.4±4.5	63.1±3.2
RGJ	64.3±4.0	55.5±9.0	56.0±7.0	59.0±4.8
ETOH	65.5±4.6	58.4±5.6	63.3±6.2	61.4±2.0

[1] Effector:target ratio 25:1
[2] A significant time-effect was observed by ANOVA independent of the type of beverage consumed ($p < 0.0001$)

effect of a single dose of RW or alcohol was studied. The rationale for such an approach was that we were interested in investigating polyphenol bioavailability from RW (Bub et al. 2001) as well as immunomodulatory effects of ethanol and polyphenols within the same study group during a 24 h period. This allowed us to relate changes in plasma polyphenol concentrations to

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potential changes in immune parameters. The volume of 500 mL RW provided the study subjects with a reasonable amount of polyphenols without exposing them to adverse ethanol concentrations.

The major polyphenol in the beverages RW, DRW, and RGJ was the anthocyanin malvidin-3-glucoside, which contributed 80% of the total anthocyanins in RW and DRW and 69% in RGJ (Bub et al. 2001). The maximum malvidin-3-glucoside plasma concentrations in our study subjects were 1–3 nM, which were reached within 120 min following beverage consumption (Bub et al. 2001). This indicates that anthocyanins were absorbed, but only low plasma concentrations were achieved, possibly too low to induce immunological effects.

In order to study whether neutrophils, monocytes and lymphocytes were specifically affected by the different beverages, cell-specific functional assays were applied. Neutrophil functions such as chemotaxis and production of reactive oxygen species are known to be affected by ethanol and polyphenols (Patel et al. 1996, Lu et al. 2001). Acute ethanol exposure in humans (5 glasses of wine or more) induced neutrophil apoptosis (Singhal et al. 1999), which is expected to impair neutrophil functions. In the present study, however, no significant effects on neutrophil phagocytosis were observed with the different beverages. A significant time-effect with phagocytic activity was measured, resulting in increased activity 3 h and 24 h after the consumption of the 4 beverages. Currently, there is no explanation for this observation other than a systemic experimental effect.

No differences in phagocytic activity and intensity of monocytes were observed between the 4 beverages suggesting that neither ethanol nor red grape-associated polyphenols were effective. *In vitro*, ethanol exposure has been shown to attenuate the phagocytic activity of human monocytes via the Fc-receptor dose-dependently (Morland and Morland 1984). While concentrations comparable to those measured in the blood of our study subjects (12 or 22 mM) had no effect *in vitro* confirming our *in vivo* observations, higher *in vitro* ethanol concentrations significantly suppressed phagocytic activity (Morland and Morland 1984).

As a monocyte-derived cytokine we measured the production of TNFα by LPS-activated monocytes. No significant treatment effects were measured; however, a significant time-effect was observed. Such a circadian rhythm for TNFα has already been reported (Petrovsky et al. 1998). In another study with acute alcohol exposure (a single intake of 80 g ethanol with wine or beer) no changes in plasma concentrations of monocyte-derived cytokines such as TNFα and IL-1α/-β were measured (Mohadjer et al. 1995). *In vitro*, only high doses (= 25 mM) as well as pharmacological doses of ethanol (> 40 mM) significantly decreased TNFα production by human monocytes (Verma et al. 1993, Szabo et al. 1996, Arbabi et al. 1999). Blood ethanol concentrations in subjects of the present study may have been too low to affect LPS-induced TNFα production.

Lymphocyte-derived cytokine production (IL-2, IL-4) was likewise not modulated by ethanol or polyphenol intake. For IL-4, a TH2-lymphocyte-specific cytokine, no differences in serum levels between controls and alcoholic patients were observed in another study (Laso et al. 1998) supporting our observations that acute ethanol consumption has no effect on TH2-lymphocytes. However, binge drinking of up to 3.1 liters of beer significantly reduced IL-2 production, a TH1-specific cytokine (Bagasra et al. 1989). In an animal study, a long-term intake of high ethanol doses significantly increased IL-2 production independent of dietary composition (Watzl et al. 1993). This suggests that, in contrast to TH2-lymphocytes, ethanol modulates TH1-lymphocytes depending on dose and intake period. Lymphocyte proliferative responsiveness to mitogen activation was also not influenced by the different beverages. This is in agreement with an earlier human study, which did not observe acute effects of ethanol after alcohol consumption (Mohadjer et al. 1995). *In vitro*, various flavonoids at a concentration of 1 μM showed no effect on ConA-activated lymphocyte proliferation of mouse splenocytes (Namgoong et al. 1993).

Lytic activity of NK cells was not affected by ETOH or by RW. Another study with acute ethanol consumption (blood ethanol levels 8.7 mM and 19.3 mM) also found no change in NK cell lytic activity (Ochshorn-Adelson et al. 1994). In contrast, chronic alcoholics without liver disease were shown to have a significantly increased number of NK cells and a parallel increase in NK cell lytic activity (Laso et al. 1997). Recently, interesting data from an animal study were published showing that long-term ethanol consumption (14% of the total daily energy intake) reduced NK cell numbers, which was not seen in mice exposed to a similar dose of ethanol taken up from red wine (Percival and Sims 2000). In this animal model red wine constituents may overcome the detrimental effects of high ethanol concentrations on NK cells. The circadian rhythm in NK cell lytic activity observed in the present study has also been reported by others (Haus 1996). Some studies have investigated the effect of wine flavonoids on NK cell function. While quercetin *in vitro* (1 mM) significantly decreased lytic activity of NK cells (no effects at lower concentrations) (Exon et al. 1998), in animal models quercetin (100 mg/kg b. w.) and catechin (125–500 mg/kg b. w.) increased lytic activity of NK cells (Ikeda et al. 1984, Exon et al. 1998). The doses used in *in vitro* studies and those obtained in the animal studies were much higher than the plasma polyphenol concentrations in the present study.

In conclusion, the results of the present study clearly

show that in healthy men acute intake of different alcoholic beverages at a volume of 500 ml has no significant immunomodulatory effects in the 24 h following beverage consumption. In contrast to the results from several *in vitro* studies (Middleton et al. 2000), a moderate polyphenol intake with RGJ and DRW did not result in immunosuppression. Whether long-term exposure to moderate amounts of alcohol and RW significantly affects immunity in man has to be investigated in future studies.

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ORIGINAL COMMUNICATION

Daily moderate amounts of red wine or alcohol have no effect on the immune system of healthy men

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Objective: To investigate whether the daily intake of red wine (RW) at a dose which inversely correlates with cardiovascular disease (CVD) risk modulates immune functions in healthy men.
Design: Randomized single-blind trial with four intervention periods.
Setting: The Institute of Nutritional Physiology, Federal Research Centre for Nutrition, Karlsruhe, Germany.
Subjects: A total of 24 healthy males with moderate alcohol consumption patterns were recruited and all completed the study.
Intervention: Participants consumed 500 ml of RW (12% ethanol (ETOH)) or 500 ml of a 12% ETOH dilution per day for a period of 2 weeks. To control the potential effects of RW polyphenols, accordingly 500 ml/day of dealcoholized red wine (DRW) and of red grape juice (RGJ) were given. The following immune parameters were measured before beverage consumption and at 1 and 2 weeks following beverage consumption: phagocytic activity of neutrophils and monocytes, production of tumor necrosis factor-alpha (TNFα), interleukin-2 and -4, transforming growth factor-β, TNFα mRNA, lymphocyte proliferation, lytic activity of natural killer cells, and percentage of apoptotic lymphocytes.
Results: Consumption of a moderate volume of alcohol with RW and with a 12% ETOH dilution had no effect on immune functions in healthy males. Consumption of polyphenol-rich beverages (DRW and RGJ) did not affect immunity-related parameters.
Conclusions: Daily moderate consumption of alcohol and of RW for 2 weeks at doses which inversely correlate with CVD risk has no adverse effects on human immune cell functions. Polyphenol-rich beverages such as RGJ and DRW further do not suppress immune responses in healthy men.
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Keywords: red wine; red grape juice; ethanol; anthocyanin; lymphocyte; immune system

Introduction

Moderate consumption of alcohol is inversely associated with cardiovascular disease (CVD) risk (Renaud *et al,* 1998; Gaziano *et al,* 1999; Rimm *et al,* 1999; Gronbaek *et al,* 2000). In addition, moderate intake of alcohol (up to three drinks) or wine may strengthen the immune response toward different types of viruses. For example, in nonsmokers intentionally exposed to rhinoviruses, alcohol consumption was associated with a decreased risk of common cold (Cohen *et al,* 1993). Epidemiological data from a recent prospective cohort study revealed that total alcohol intake as well as beer and spirit consumptions were not related to the occurrence of common cold, whereas moderate consumption of wine was inversely associated with the risk of common cold. The association was stronger for red wine (RW) when compared with white wine (Takkouche *et al,* 2002), which suggests that specific polyphenols in RW may contribute to the observed reduction in common cold risk. Recently, in an animal study it was shown that ethanol (ETOH) intake with RW (corresponding to an intake of 360 ml of RW in humans) produced no changes in blood lymphocyte and NK cell numbers, while a similar intake of ETOH alone reduced the percentage of these cells (Percival & Sims, 2000). Based on these data, one may speculate that polyphenols in RW protect from potential adverse effects of ethanol on the immune system.

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Besides its beneficial effect on CVD risk, alcohol consumption at a level of more than three drinks per day is associated with an increased all-cause mortality (Ellison, 2002). Chronic ethanol abuse is known to result in specific defects in innate and acquired immunity and may increase host susceptibility to infections (Watzl & Watson, 1992; Nelson & Kolls, 2002). In a case–control study of patients with community-acquired pneumonia, alcohol intake was identified as a risk factor. High-alcohol intake patients showed a higher incidence of pneumonia and more severe clinical symptoms than patients with a low alcohol intake (Fernandez-Sola *et al,* 1995). ETOH *in vitro* suppresses significantly the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) by human peripheral blood mononuclear cells (PBMCs) (Watzl & Watson, 1993; Szabo *et al,* 1996). Chronic alcohol intake, however, results in an enhanced response of proinflammatory cytokines (Nelson & Kolls, 2002). Moreover, ETOH exposure of polymorphonuclear neutrophilic granulocytes (PNG) and monocytes *in vitro* and *in vivo* inhibits phagocytosis (Stoltz *et al,* 1999) and decreases their capacity to produce reactive oxygen species during the oxidative burst (Patel *et al,* 1996; Szabo, 1999).

Alcohol consumption and its subsequent metabolism in the liver generate reactive oxygen species, which interfere with various immune cell functions. Phenolic antioxidants in RW may scavenge reactive oxygen species and thereby protect the host against impairment of immune cell functions by ETOH intake. RW consumption delivers polyphenols such as the flavonoids with strong *in vitro* and *in vivo* antioxidant (Wang *et al,* 1997; Casalini *et al,* 1999; Lodovici *et al,* 2001) and immunomodulatory effects (Middleton *et al,* 2000). Despite this, the immunological effects of flavonoids in humans have not yet been studied. Most polyphenol studies so far were done in *in vitro* systems demonstrating that flavonoids suppress a variety of immune functions including lymphocyte proliferation, lytic activity of NK cells, and cytokine secretion (Middleton *et al,* 2000).

The objective of this study was first to investigate whether daily consumption of a moderate volume of RW modulates immune functions in healthy men during a period of 2 weeks. The second aim was to investigate whether polyphenols in RW with their antioxidative and immunomodulatory potential induce changes in immune functions that differ from those induced by the consumption of a 12% ETOH beverage. To broadly assess changes in immune functions, we have measured a large range of immune parameters.

Subjects and methods

Subjects

In all, 24 nonsmoking men with moderate alcohol consumption patterns ($\leq$80 g ETOH/week) and with normal body weight were recruited for the study. All subjects were in good medical health as determined by a screening history and medical examination. None were taking vitamin supplements or medications 2 months before or during the study. The study was approved by the Medical Ethical Committee of the Landesärztekammer Baden-Württemberg and all participants gave their consent in writing.

Study design

This study was a randomized crossover study of four experimental treatments, each lasting 14 days. During the experimental treatments, subjects were randomly assigned to consume 500 ml/day of RW (12% ETOH v/v), dealcoholized red wine (DRW), red grape juice (RGJ), and ETOH (12% ethanol v/v), with a wash-out period of 1 week between each experimental treatment. Subjects were instructed to drink the beverages with their main meals. During the study period, subjects adhered to their usual diets, but were instructed to avoid alcohol-containing beverages and food products rich in anthocyanins or polyphenols (Bub *et al,* 2001).

RW (variety Lemberger) and RGJ (variety Lemberger) grown in the same vineyard were obtained from the State Winery Weinsberg (Baden-Württemberg, Germany). Dealcoholization of the RW was achieved by a vacuum rectification process (Centre for dealcoholization EAZ Petershans GmbH, Waiblingen, Germany). The total anthocyanin (mg/l) and total catechin ((+)-catechin and (−)-epicatechin, mg/l) contents were 171.1 and 63.4 (RW), 144.8 and 47.0 (DRW), and 338.6 and 64.3 (RGJ), respectively. The resveratrol content (mg/l) was 4.94 (RW), 5.15 (DRW), and 3.75 (RGJ) (Bub *et al,* 2001). The 12% ethanol beverage was prepared from vodka.

Collection and preparation of blood samples

Fasting venous blood samples were collected once a week between 7:00 and 10:00. Blood was drawn from an antecubital vein into prechilled tubes containing Li-heparin (Monovette-Sarstedt, Nümbrecht, Germany) and immediately placed on ice in the dark. Plasma was collected after centrifugation at $1500 \times g$ for 10 min at 4°C. PBMC were prepared as described earlier (Watzl *et al,* 2000).

Lymphocyte proliferation

PBMC at 1×10^9 cells/l in a medium containing 10% of FBS were stimulated by the T-cell mitogen concanavalin A (5 mg/l, ConA, Sigma) for 120 h at 37°C. Proliferation was measured using the pyrimidine analog 5-bromo-deoxyuridine, which was quantified in PBMC by a cellular enzyme immunoassay as described earlier (Watzl *et al,* 2000).

Quantification of cytokine secretion

PBMC at 1×10^9 cells /l were cultured in medium containing 10% of FBS and stimulated by 5 mg/l ConA for 24 h at 37°C

(interleukin-2, -4 IL-2, IL-4) or by 1 μg/l LPS (TNFα, transforming growth factor-β, TGFβ). Cell-free supernatants were collected and stored at −80°C until analysis. TNFα, IL-2, and IL-4 were measured by sandwich-ELISAs as described earlier (Watzl *et al,* 2000). For TGFβ, a sandwich-ELISA was developed using an anti-human TGFβ monoclonal antibody (2 mg/l PBS, pH 7.4; R&D, Wiesbaden, Germany) as capture antibody and a monoclonal biotin-labeled mouse anti-human TGFβ antibody (300 μg/l reagent diluent, R&D) as detection antibody. TGFβ in supernatants had to be activated by mixing 0.5 ml of supernatants with 0.1 ml 1 M HCl. After incubation for 10 min, 0.1 ml of 1.2 M NaOH/0.5 M HEPES was added, then the mixture was pipetted on a microtiter plate. Optical density was measured with a multiplate spectrophotometer (Molecular Devices) at 405 nm with data expressed as nanogram per liter compared to the optical density of recombinant TGFβ.

Quantification of TNFα mRNA

PBMCs were LPS-stimulated under conditions as already described for the ELISA-based TNFα analysis, except that duration of LPS-incubation for mRNA detection was 4 h. Total RNA was extracted from the cells using the guanidine isocyanate/acid phenol method described by Chomczynski and Sacchi (1987). The quantity and quality of the RNA were measured at 260/280 nm in a spectrophotometer (Lamda Bio 20 UV/VIS, Perkin-Elmer, Wellesley, MA, USA).

RT–PCR

Reverse transcription (RT) and polymerase chain reaction (PCR) were performed using the Ready-to-Go RT–PCR kit (Amersham Pharmacia Biotech; Freiburg, Germany). The respective sequences used for generation of sense and antisense primers were nt 350–373 and nt 795–772 of TNFα cDNA (Acc. XM_165823.1) and nt 71–94 and nt 570–574 of GAPDH cDNA (Acc. BC004109) used as internal standard for normalization. All primers were tested for linearity over cycle number, and analyses were all carried out in the linear portion of the curve, therefore allowing semiquantitative analysis of mRNA amount. PCR products were run on 1.5% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.9) and ethidium bromide stained bands were visualized and quantified using computer-based image analysis (FluorS Imager; Biorad, München, Germany). Quantities of each PCR product were normalized by dividing the average gray level of the signal by that of the corresponding GAPDH PCR product. Data are expressed as arbitrary units (AU).

Lytic activity of NK cells

Lytic activity of NK cells against K562 target cells (effector:-target ratios 50:1, 25:1, 12.5:1) was measured with a recently described flow cytometric method (Watzl *et al,* 2000).

Phagocytic activity

Assessment of phagocytic activity (percentage of phagocytic-active cells) was based on a recently described flow cytometric method (O'Gorman, 2002).

Apoptosis

Apoptosis was measured in PBMC using Annexin V with a recently described flow cytometric method (Roser *et al,* 2001).

Statistical analyses

Subjects were randomly assigned to a different treatment order regarding the four beverages. As a consequence, only three subjects consumed all beverages in the same sequence during the four phases of beverage consumption. To test whether baseline data for one beverage were significantly different in the four different phases, data were analyzed by factorial ANOVA with 'time' as dependent variable and 'beverage' and 'phase' as factors. When no significant differences between baseline data of the four phases for one beverage were observed, data of the four phases were combined to one experimental period resulting in a total number of 24 subjects with blood collections at weeks 0, 1, and 2. Repeated-measures ANOVA were used with 'time' as within-subject factor and 'beverage' as between-subject factor to analyze treatment effects. Results are reported as means ± standard error of the mean (s.e.m.). All statistical calculations were performed with the StatView program (SAS Institute 1998, Cary, NC, USA).

Results

Mean age of the study subjects was 30.6 ± 1.4 y. Body mass index (kg/m^2) and body mass (kg) were 23.5 ± 0.4 and 79.7 ± 1.4, respectively. All participants tolerated the intervention with the four beverages well and completed the study. Maximum blood ethanol concentrations in subjects consuming red wine were 15.9 and 15.0 mM in subjects consuming the 12% ETOH dilution (Watzl *et al,* 2002).

No beverage-specific differences at baseline for the four different phases were observed. Therefore, data of the four phases for one beverage were combined into one phase of beverage consumption. The capacity of mitogen-activated PBMC to produce TH1-(IL-2) and TH2-(IL-4) lymphocyte-specific cytokines as well as monocyte-specific cytokines (TGFβ, TNFα) did not change significantly over time when the four different beverages were compared (Table 1). In order to study the effect of ETOH on cytokine gene expression, we measured TNFα mRNA expression. No beverage-specific effects on TNFα mRNA were observed (Table 1). Phagocytic activity of granulocytes and monocytes *ex vivo* was not different at baseline and was not significantly affected by the consumption of the different beverages (Table 2). Apoptosis of T lymphocytes, T-lymphocyte

Table 1 Production of IL-2, IL-4, TGFβ, TNFα, and TNFα mRNA expression by activated PBMCs isolated from subjects consuming for 14 days a volume of 500 ml/day red wine (RW), red grape juice (RGJ), dealcoholized red wine (DRW), or a 12% ethanol control beverage (ETOH) (n=24, mean$\pm$s.e.m.)

Treatment	*RW*			*RGJ*			*DRW*			*ETOH*		
Week	*0*	*1*	*2*	*0*	*1*	*2*	*0*	*1*	*2*	*0*	*1*	*2*
IL-2 (ng/l)	877$\pm$67	990$\pm$117	970$\pm$100	972$\pm$90	1023$\pm$92	991$\pm$87	899$\pm$79	982$\pm$83	983$\pm$77	943$\pm$94	964$\pm$98	927$\pm$95
IL-4 (ng/l)	38$\pm$4	38$\pm$3	40$\pm$4	42$\pm$6	45$\pm$5	44$\pm$6	38$\pm$4	41$\pm$4	50$\pm$6	40$\pm$5	40$\pm$4	42$\pm$5
TGFβ (ng/l)	1501$\pm$76	1355$\pm$96	1332$\pm$69	1345$\pm$108	1390$\pm$66	1446$\pm$73	1401$\pm$74	1323$\pm$77	1367$\pm$72	1369$\pm$83	1369$\pm$107	1346$\pm$526
TNFα (ng/l)	811$\pm$41	763$\pm$34	786$\pm$29	785$\pm$31	770$\pm$38	756$\pm$43	750$\pm$43	741$\pm$37	751$\pm$38	783$\pm$41	723$\pm$35	772$\pm$45
TNFα-mRNA (AU)	1.4$\pm$0.2	1.5$\pm$0.1	1.3$\pm$0.2	1.4$\pm$0.2	1.4$\pm$0.1	1.0$\pm$0.1	1.0$\pm$0.2	1.3$\pm$0.2	1.1$\pm$0.1	1.2$\pm$0.1	1.3$\pm$0.2	1.1$\pm$0.1

Table 2 Immune parameters measured *ex vivo* with PBMCs isolated from subjects consuming for 14 days a volume of 500 ml/day of red wine (RW), red grape juice (RGJ), dealcoholized red wine (DRW), or a 12% ethanol control beverage (ETOH) (n=24, mean$\pm$s.e.m.)

Treatment	*RW*			*RGJ*			*DRW*			*ETOH*		
Week	*0*	*1*	*2*	*0*	*1*	*2*	*0*	*1*	*2*	*0*	*1*	*2*
Phagocytosis (%)												
Granulocytes	46$\pm$2	38$\pm$3	44$\pm$3	45$\pm$2	41$\pm$2	41$\pm$3	42$\pm$3	39$\pm$3	44$\pm$3	45$\pm$2	37$\pm$2	45$\pm$3
Monocytes	32$\pm$2	26$\pm$2	28$\pm$2	27$\pm$2	25$\pm$1	26$\pm$2	27$\pm$2	25$\pm$2	27$\pm$2	30$\pm$2	24$\pm$2	28$\pm$2
Apoptosis (%)												
Lymphocytes	8.2$\pm$0.4	6.5$\pm$0.3	7.5$\pm$0.4	7.1$\pm$0.3	7.2$\pm$0.4	7.2$\pm$0.4	7.7$\pm$0.4	7.1$\pm$0.4	7.0$\pm$0.4	7.3$\pm$0.4	7.2$\pm$0.3	6.8$\pm$0.4
Lymphocyte												
Proliferation (A_{450}–A_{650})	1.8$\pm$0.06	1.7$\pm$0.06	1.7$\pm$0.05	1.8$\pm$0.06	1.8$\pm$0.05	1.7$\pm$0.05	1.7$\pm$0.06	1.7$\pm$0.06	1.7$\pm$0.05	1.7$\pm$0.07	1.7$\pm$0.06	1.7$\pm$0.07
NK cell activity (%)[a]	52$\pm$3	54$\pm$3	56$\pm$3	52$\pm$3	54$\pm$3	54$\pm$3	51$\pm$3	53$\pm$4	55$\pm$4	48$\pm$3	50$\pm$3	51$\pm$3

[a]Effector:target ratio 25:1.

responsiveness to mitogen-activation and lytic activity of NK cells (data are only shown for the effector:target ratio of 25:1) were also not modulated by the 2 weeks of beverage consumption (Table 2).

Discussion

We have recently shown that the acute intake of moderate amounts of red wine or 12% ETOH has no effect on the immune system of healthy men in the following 24 h (Watzl *et al,* 2002). The objective of the present study was to investigate the immunomodulatory effects of a daily moderate intake of RW, DRW, RGJ and of 12% ETOH over a period of 2 weeks. In order to study whether neutrophils, monocytes, and lymphocytes were specifically affected by the different beverages, cell-specific functional assays were applied. Neutrophil functions such as chemotaxis and production of reactive oxygen species have previously shown to be affected by ethanol and polyphenols (Patel *et al,* 1996; Lu *et al,* 2001). Acute ETOH exposure in humans (five glasses of wine or more) induced neutrophil apoptosis (Singhal *et al,* 1999a), which is expected to impair neutrophil functions. In the present study, however, no significant effects on neutrophil as well as monocyte phagocytosis were observed with the different beverages suggesting that neither ETOH nor red grape-associated polyphenols were effective. *In vitro*, ETOH exposure has been shown to attenuate dose-dependently the phagocytic activity of human monocytes via the Fc-receptor (Morland & Morland, 1984). While ETOH concentrations (12 or 22 mM) comparable to those measured in the blood of our study subjects (15.9 mM; Watzl *et al,* 2002) also had no effect *in vitro* confirming our *in vivo* observations, higher *in vitro* ETOH concentrations significantly suppressed phagocytic activity (Morland & Morland, 1984).

The production of TNFα by LPS-activated monocytes as well as TNFα mRNA expression were not significantly affected by the treatment. In another study with acute alcohol exposure (a single intake of 80 g ethanol with wine or beer), no changes in plasma concentrations of monocyte-derived cytokines such as TNFα and IL-1α/-β were measured (Mohadjer *et al,* 1995). *In vitro*, only high, but still physiological doses ($\geq$25 mM) as well as pharmacological doses of ETOH ($>$40 mM) significantly decreased TNFα production by human monocytes (Verma *et al,* 1993; Szabo *et al,* 1996; Arbabi *et al,* 1999). In addition, a concentration of 25 mM ETOH *in vitro* also reduced TNFα mRNA levels

(Szabo *et al,* 1996). Blood ETOH concentrations in subjects of the present study may not have been high enough to affect LPS-induced TNFα production at the protein and at the mRNA level.

ETOH or polyphenol intake did not modulate lymphocyte-derived cytokine production (IL-2, IL-4). For IL-4, a TH2-lymphocyte-specific cytokine, no differences in serum levels between controls and alcoholic patients were observed in another study (Laso *et al,* 1998) supporting our observations that daily ETOH consumption at this level has no effect on TH2-lymphocytes. However, binge drinking of up to 3.1 l of beer significantly reduced IL-2 production, a TH1-specific cytokine (Bagasra *et al,* 1989). In an animal study, a long-term intake of high ETOH doses significantly increased IL-2 production independent of the dietary composition (Watzl *et al,* 1993). This suggests that in contrast to TH2-lymphocytes ethanol modulates TH1-lymphocytes depending on dose and length of intake period. Lymphocyte proliferative responsiveness to mitogen-activation was also not influenced by the different beverages. This is in agreement with an earlier human study, which did not observe acute effects of ethanol after alcohol consumption (Mohadjer *et al,* 1995).

In the present study, lytic activity of NK cells was not affected by ETOH or by RW. Another study with acute ETOH consumption (blood ETOH levels 8.7 and 19.3 mM) also did not observe a change in NK cell lytic activity (Ochshorn-Adelson *et al,* 1994). In contrast, chronic alcoholics without liver disease were shown to have a significantly increased number of NK cells and a parallel increase in NK cell lytic activity (Laso *et al,* 1997). Several studies have investigated the effect of wine flavonoids on NK cell function. While quercetin *in vitro* (1 mM) significantly decreased lytic activity of NK cells (no effects at lower concentrations) (Exon *et al,* 1998), in animal models, quercetin (100 mg/kg) and catechin (125–500 mg/kg) increased lytic activity of NK cells (Ikeda *et al,* 1984; Exon *et al,* 1998). The doses used in *in vitro* studies and those obtained in the animal studies were much higher than the plasma flavonoid concentrations in our study subjects after a single intake of these beverages. The major polyphenol in the beverages RW, DRW, and RGJ was the anthocyanin malvidin-3-glucoside, which contributed 80% of the total anthocyanins in RW and DRW and 69% in RGJ (Bub *et al,* 2001). The maximum malvidin-3-glucoside plasma concentrations were 1–3 nM, which were reached within 120 min following beverage consumption (Bub *et al,* 2001). This indicates that the major polyphenol from red wine was absorbed, but only low quantities were available. Whether these plasma concentrations are significant to induce physiological activity can only be speculated at present.

Results from recent studies reported that acute alcohol intake (five glasses of wine) enhanced the percentage of apoptotic neutrophils and monocytes (Singhal *et al,* 1999a, b). So far, no information was available on the percentage of lymphocyte apoptosis after moderate ETOH or polyphenol intake. The present data show that neither RW nor 12% ETOH at a volume of 500 ml/day affected lymphocyte apoptosis. TGF-β production by LPS-activated monocytes, which has been demonstrated to partly mediate the increase in monocyte apoptosis by ETOH (Singhal *et al,* 1999b), also did not differ between the beverages.

In conclusion, the results of the present study clearly show that the daily intake of different alcoholic beverages at a volume of 500 ml over a period of 2 weeks had no significant immunomodulatory effects. In addition, a high polyphenol intake with RGJ and DRW did not result in immunosuppression as suggested by results from several *in vitro* studies (Middleton *et al,* 2000). These data indicate that daily RW consumption at a level, which inversely correlates with CVD risk, has no adverse effects on the immune system. Whether longer time periods with such alcohol consumption patterns result in more significant changes of immune functions cannot be answered by the present study.

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Prebiotic Inulin Enriched with Oligofructose in Combination with the Probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* Modulates Intestinal Immune Functions in Rats[1]

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ABSTRACT Probiotics (PRO) modulate systemic immunity in animals and humans. In contrast, the effects of prebiotics (PRE) on systemic and intestinal immunity have not been investigated. Whether the combined application of PRO and PRE [synbiotics (SYN)] has synergistic or additive effects is presently unknown. Therefore, PRO (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12), PRE (inulin enriched with oligofructose), and SYN (combination of PRO and PRE) were fed to F344 rats for 4 wk as supplements to a high fat diet. Functions of immune cells isolated from peripheral blood mononuclear cells (PBMC), spleen, mesenterial lymph nodes and Peyer's patches (PP) were investigated. The SYN supplement increased secretory immunoglobulin A (sIgA) production in the ileum compared with controls fed the high fat diet alone ($P < 0.05$), and decreased the oxidative burst activity of blood neutrophils ($P < 0.05$) compared with rats fed PRO. The PRE supplement enhanced the production of interleukin-10 ($P < 0.05$) in PP as well as the production of sIgA in the cecum ($P < 0.05$), compared with controls. The PRO supplement modestly affected immune functions, whereas systemic immunomodulatory effects were observed in rats fed SYN. The PRE supplement primarily acted at the level of the gut-associated lymphoid tissue. The combined application of PRO and PRE has different effects from those of the individual supplements, but does not simply result in additive or synergistic effects. J. Nutr. 134: 153–156, 2004.

KEY WORDS: • Immune sytem • gut • probiotic • prebiotic • synbiotic • rat

Several recent reviews summarized the available experimental evidence for the immunomodulatory effects of probiotics (PRO)[4] (1,2). Although experimental and human studies clearly show that PRO affect host resistance to intestinal infections as well as a number of immune cell functions, the results of these studies vary greatly. In addition, the underlying immunological mechanisms of PRO are generally not well defined (3,4). The gut-associated lymphoid tissue (GALT) is increasingly recognized as a crucial component of immune response (5). Although many studies have focused on the systemic immunological effects of PRO, few data are available regarding systemic immunological effects and local immunological effects in the gut in the same animal (6). Therefore, studies investigating the immunomodulatory potential of PRO, including their effects at the GALT level, are needed.

At present, few studies have investigated the direct effects of prebiotics (PRE) on the immune system (7). Whether PRE modulate the immune response directly or indirectly, by affecting the composition of the intestinal flora and thus affecting the GALT, or by producing SCFA, is presently unknown.

Our study investigated the effects of PRO, PRE and their combined application [synbiotics (SYN)] at the systemic level and at the GALT level. We hypothesized that PRO and PRE, alone or in combination, would modify immune responses in the GALT, enhancing cytokine production and other immune cell functions. Therefore, we fed F344 rats consuming a high fat, low fiber control diet a daily supplement consisting of two probiotic strains (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12), an inulin-based prebiotic enriched with oligofructose or the combination of PRO and PRE (SYN) for a period of 4 wk. We decided to use two probiotic strains because others have suggested that a mixture of PRO may have a greater effect on the intestine than the individual strains (8,9). We assessed a broad spectrum of immune functions with cells isolated from peripheral blood mononuclear cells (PBMC) and the spleen as well as from the GALT. We used a Western-style high fat, low fiber diet instead of a standard rat diet as the control diet to make the study conditions more comparable to the situation of humans in Western countries consuming PRO and/or PRE.

MATERIALS AND METHODS

Animals. The State Veterinary Office granted permission for the rat studies, and the experiments complied with its guidelines for the care and use of laboratory animals. Male Fischer 344/NHsd rats (Harlan Winkelmann, Borchen, Germany) aged 12 to 13 wk were fed a standard lab diet (Altromin, Lage, Germany) for 1 wk, and all rats were then fed the control diet for 1 wk.

Diets. Dietary components were purchased from Piccioni (Gessate, Milan, Italy). RaftiloseR Synergy1 was provided by Orafti (Tienen, Belgium). This PRE is an oligofructose-enriched inulin,

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[4] Abbreviations used: Bb12, *Bifidobacterium lactis* Bb12; BSA, bovine serum albumin; CFU, colony-forming units; ConA, concanavalin A; GALT, gut-associated lymphoid tissue; HF, high fat; LAB, lactic acid bacteria; LGG, *Lactobacillus rhamnosus* GG; MLN, mesenteric lymph nodes; NK, natural killer; PBMC, peripheral blood mononuclear cells; PP, Peyer's patches; PRE, prebiotics; PRO, probiotics; PTX, pentoxifylline; sIgA, secretory immunoglobulin A; SYN, synbiotic.

comprised of a 1:1 mixture of long- and short-chain fractions of inulin, a β(2–1)-fructan extracted from chicory roots (*Cichorium intybus*). *L. rhamnosus* GG (LGG) and *B. lactis* Bb12 (Bb12) were provided by Valio (Helsinki, Finland) and purchased from Chr. Hansen (Horsholm, Denmark), respectively. They were supplied as freeze-dried powder in sealed packets containing $\sim 6 \times 10^{11}$ colony-forming units (CFU)/g (LGG) and $\sim 3 \times 10^{10}$ CFU/g (Bb12), respectively. The bacteria were stored at a temperature of −20°C until used. The dietary concentration of LGG and Bb12 was evaluated as described elsewhere (10).

Rats ($n = 80$) were allocated to four experimental groups ($n = 20$). The control group was fed a high fat (HF) diet based on the AIN76 diet (11), modified to contain a high level of fat (231 g corn oil/kg) and a low level of cellulose (20 g/kg) to compare with the diet typical of Western humans at high risk of colon cancer (10). The sources of carbohydrates in this diet were sucrose (361 g/kg) and maltodextrins (100 g/kg). The PRO group was fed an HF diet supplemented with LGG and Bb12 to provide $\sim 5 \times 10^{11}$ CFU of each strain per kg of diet. The PRE group was fed an HF diet with the maltodextrins replaced by 100 g/kg of Raftilose Synergy1. The SYN group was fed the PRE-group diet supplemented with LGG and Bb12 to provide $\sim 5 \times 10^{11}$ CFU of each strain per kg of diet, matching the levels in the PRO-group diet. Food and water were consumed ad libitum. Diets were prepared every week, divided into aliquots and frozen at −20°C. At the end of the 4-wk experimental feeding period, the rats were anesthetized with CO_2 and then decapitated.

Preparation of immune cell suspensions and fecal samples. Immune cell suspensions were prepared with RPMI-1640 culture medium containing 50 mL/L of heat-inactivated fetal bovine serum, L-glutamine (2 mmol/L), penicillin (1×10^5 U/L), streptomycin (100 mg/L) and HEPES (25 mmol/L); all components were purchased from Life Sciences (Eggenstein-Leopoldshafen, Germany). Trunk blood was collected in heparinized tubes after decapitation. The PBMC and splenocytes were isolated as previously described (12). The intestinal contents of the ileum and cecum were collected and stored at −20°C until assayed.

Fluorescence staining of lymphocyte subpopulations. The expression of cell surface markers on the immune cells of blood, spleen and mesenteric lymph nodes (MLN) was investigated by immunofluorescence as previously described (12). Phycoerythrin-conjugated mouse anti-rat monoclonal antibodies to CD4 (Caltag, Hamburg, Germany) and fluorescein-conjugated mouse anti-rat monoclonal antibodies to CD8 (Caltag) with appropriate isotype controls (Caltag) were used. Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Phagocytosis. The phagocytic capacity of the immune cells of the blood and the spleen was assessed with a flow cytometric method as previously described (13).

Oxidative burst. Whole blood (100 μL) was cooled on ice to 0°C. Tubes were incubated with 20 μL PBS (control) or 20 μL phorbol myristate 13-acetate (8.1 μmol/L; Sigma-Aldrich, Deisenhofen, Germany), as a high stimulus for 10 min at 37°C. Dihydrorhodamine 123 (1.7 μL, 29 mmol/L; Molecular Probes, Leiden, Netherlands) was added and samples were incubated for 15 min at 37°C. Erythrocytes were lysed with 2 mL 1× lysing solution. After centrifugation (250 × g, 4°C, 5 min), cells were washed with 3 mL PBS. The cells were resuspended in 300 μL propidium iodide (0.75 μmol/L; Sigma-Aldrich) and incubated for 10 min on ice. Samples were analyzed within 30 min with the flow cytometer.

Cytotoxicity of natural killer (NK) cells. The natural killer (NK) cell activity of the immune cells of the blood, spleen, MLN and PP was assessed with a flow cytometric method as previously described (14), using target cells from the mouse Moloney leukemia cell line YAC-1.

Lymphocyte proliferation. The ex vivo proliferative responsiveness of lymphocytes isolated from the spleen, MLN (each 5×10^9 cells/L) and PP (7×10^9 cells/L) to the mitogen concanavalin A (ConA; 1 mg/L; Sigma-Aldrich, Deisenhofen, Germany) for 72 h at 37°C, 5% CO_2 and 95% humidity was determined by ELISA, using a commercial proliferation kit (Cell Proliferation ELISA kit; Roche Diagnostics, Mannheim, Germany).

Cytokines. Splenocytes (100 μL; 1×10^9 cells/L IFN-γ and 5×10^9 cells/L IL-10), MLN (100 μL; 1×10^9 cells/L IFN-γ and 5×10^9 cells/L IL-10) and PP (100 μL; 5×10^9 cells/L IFN-γ and 5×10^9 cells/L IL-10) were stimulated by 100 μL of Con A (5 mg/L) for 24 h at 37°C, 5% CO_2 and 95% humidity. Levels of IFN-γ and IL-10 in the supernatants were measured with OptEIA commercial ELISA kits (BD Pharmingen, Heidelberg, Germany), following the manufacturers' instructions.

Secretory immunoglobulin A (sIgA). Fresh caecal (140 mg/rat) and ileal contents (10 mg/rat) were mixed with 1 mL of a solution (10 g/L) of bovine serum albumin (BSA; Sigma-Aldrich) in PBS and incubated for 10 min at room temperature. Samples were centrifuged (4000 × g, 30 min, 20°C) and supernatants were collected and stored at −20°C until assayed. Maxisorb 96-well microtiter plates (NUNC, Roskilde, Denmark) were coated with 100 μL of a rabbit anti-rat secretory component antibody (Bethyl Laboratories, Montgomery, AL) diluted 1:1000 with a solution (50 g/L) of Tween 20 (Sigma) in PBS and incubated overnight at 4°C. After blocking with 100 μL of BSA (40 g/L) in the Tween solution for 1 h and washing, 100-μL titers of the samples were applied. After incubation for 2 h, 100 μL of horseradish peroxidase-conjugated goat monoclonal anti-rat IgA (Bethyl Laboratories) diluted 1:500 with the Tween-BSA solution was added, and the plates were then incubated for 2 h. A peroxidase substrate, 100 μL of tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was then added, and the plates were incubated for 30 min in the dark. The enzyme reaction was stopped with 100 μL of 1 mol/L H_3PO_4. The relative amount of secretory immunoglobulin A (sIgA) was quantified by measuring the absorbance at 450 nm.

Statistics. Results are reported as means ± SD. Differences among groups were tested for significance by one-way ANOVA with the Tukey-Kramer test for comparison of individual means when appropriate. To assess correlations between single immunological markers, Pearson correlation coefficients were computed. Values of $P < 0.05$ were considered significant. All statistical calculations were performed with the StatView program (1998 release, SAS Institute, Cary, NC).

RESULTS

Supplementation of the control diet with PRO, PRE and SYN for 4 wk did not affect feed intake(15.0 ± 1.05 g/d), weight gain (46 ± 11 g), and final body weight (370 ± 21 g). The intake of LGG was $\sim 7.3 \pm 0.5 \times 10^9$ CFU/d, and intake of Bb12 was $7.6 \pm 0.6 \times 10^9$ CFU/d.

No significant differences in $CD4^+$ and $CD8^+$ T-lymphocytes were observed among the treatment groups in any of the tissues studied (data not shown). In the blood the CD4:CD8 ratio tended to be greater ($P = 0.08$) in rats treated with PRO or SYN than in the control or PRE groups (control, 1.80 ± 0.22; PRE, 1.81 ± 0.16; PRO, 1.95 ± 0.35; SYN, 1.95 ± 0.21).

Neutrophil and monocyte phagocytosis (percentage active cells and mean fluorescence intensity) were not affected by PRO, PRE or SYN (data not shown). Oxidative burst activity was significantly reduced in blood neutrophils isolated from rats treated with SYN compared with rats treated with PRO (**Table 1**).

The treatments tended ($P = 0.09$) to affect NK cell activity in PBMC (control, 15 ± 6%; PRE, 16 ± 5%; PRO, 18 ± 6%; SYN, 19 ± 6%). The NK cell activity did not differ among the groups in any other tissue studied. Lymphocyte proliferation was not affected by the dietary treatments in any of the tissues studied (data not shown).

The treatments did not modulate cytokine production in the spleen or MLN (data not shown), but PRE treatment increased IL-10 production in PP relative to the control group ($P < 0.05$; **Table 2**). The production of IFN and IL-10 in this tissue were correlated ($r = 0.90$; $P < 0.0001$).

TABLE 1

Oxidative burst activity in peripheral blood neutrophils of rats fed a high fat diet supplemented with a probiotic (PRO), a prebiotic (PRE) and a synbiotic (SYN)[1]

Parameter	Control	PRO	PRE	SYN
Active cells, %	41 ± 14	40 ± 13	40 ± 10	38 ± 11
Fluorescence intensity	47 ± 5^{ab}	48 ± 7^{a}	44 ± 6^{ab}	42 ± 6^{b}

[1] Values are means ± SD, $n = 20$. Means in a row without a common letter differ, $P < 0.05$.

The SYN treatment enhanced the sIgA concentration in the ileum ($P < 0.05$), and the PRE treatment increased it in the cecum ($P < 0.05$; **Table 3**), relative to the control group.

DISCUSSION

The objectives of the present study were to investigate whether the effects of PRO and PRE on the immune system vary among different immune compartments and whether SYN has a greater effect than that obtained with PRO or PRE supplementation alone. The results demonstrate that PBMC and PP are the primary tissues that are specifically affected by PRE. In addition, although PRE supplementation alone induced significant immunomodulation in the intestine, PRO supplementation was primarily effective when provided as a component of SYN.

We used a high fat, low-fiber diet instead of a standard rat diet because it more closely resembles the dietary conditions of Western humans consuming PRO and/or PRE. The fat content of the diet affects immune functions such as NK cell activity (15). Therefore, the potential immunomodulatory effects of PRO and PRE may become more obvious in subjects fed such a diet.

Overall, the treatments did not affect the subpopulations of lymphocytes in the blood, spleen and MLN. However, the PRO and SYN treatments tended to increase the CD4:CD8 ratio in the blood. This suggests that LGG + Bb12 treatment modulates the composition of circulating lymphocytes in the periphery. In another study with rats, consuming yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) for 4 wk did not affect lymphocyte subpopulations in the PBMC and spleen when compared with unfermented milk (6). In mice, consuming lactic acid bacteria (LAB) for 4 wk did not affect the percentages of $CD4^+$ and $CD8^+$ cells in the blood (16).

TABLE 2

Cytokine production of Peyer's patch (PP) cells of rats fed a high fat diet supplemented with a probiotic (PRO), a prebiotic (PRE) and a synbiotic (SYN)[1,2]

Cytokine	Control	PRO	PRE	SYN
		μg/L		
IFN	2.7 ± 0.8	2.7 ± 1.0	3.6 ± 2.0	2.9 ± 1.2
IL-10	0.31 ± 0.13^{a}	0.37 ± 0.19^{ab}	0.56 ± 0.38^{b}	0.46 ± 0.29^{ab}

[1] Values are means ± SD, $n = 20$. Means in a row without a common letter differ, $P < 5$.

[2] Lymphocytes were stimulated with 5 mg/L concanavalin A (ConA) and cultivated for 24 h.

TABLE 3

Secretory immunoglobulin A (sIgA) concentrations in ileum and cecum of rats fed a high fat diet supplemented with a probiotic (PRO), a prebiotic (PRE) and a synbiotic (SYN)[1]

Tissue	Control	PRO	PRE	SYN
		OD_{450}		
Ileum	0.78 ± 0.36^{a}	0.92 ± 0.41^{ab}	1.09 ± 0.62^{ab}	1.30 ± 0.75^{b}
Caecum	0.66 ± 0.42^{a}	0.88 ± 0.59^{ab}	1.15 ± 0.77^{b}	0.72 ± 0.31^{ab}

[1] Values are means ± SD, $n = 20$. Means in a row without a common letter differ, $P < 0.05$.

Altogether these data suggest that PRO and PRE have only minor effects on the composition of T cell subsets in different immune compartments.

In contrast to a number of animal and human studies (17), the present study found that PRO treatment did not stimulate neutrophil and monocyte phagocytosis. A study with humans also found that *Lacobacillus casei* Shirota supplementation for 4 wk did not affect phagocytosis in healthy adults (18). Because the subjects of that study also consumed a high fat diet comparable to the one used in the present study with rats, the basic diet might be at least partly responsible for the observed differences. The reason for the significant reduction of the oxidative burst activity in the SYN group is unknown.

In the present study, lymphocytes from the blood, spleen and GALT, including PP and MLN, exerted an NK-like cytotoxic effect (based on their capacity to lyse the YAC-1 target cell line). Because no earlier study reported NK cell activity in rat PP, we used pentoxifylline (PTX), a suppressor of NK cell activity (19), to suppress NK cell activity in the spleen and PP. The PTX suppressed NK cell activity in both tissues (data not shown).

The PRO and SYN treatments tended to enhance NK cell activity only in blood. This suggests that LGG + Bb12 supplementation may have caused this increase. Studies in humans have demonstrated that *L. rhamnosus* (strain HNOO1) and *B. lactis* (strain HN019) supplementation upregulate PBMC NK cell cytotoxicity (17), although the overall nutritional status of the study subjects also modulated the effect of this PRO treatment (20). These data support the observations of the present study with rats. Supplementation with *B. lactis* in oligosaccharide-enriched milk caused higher NK cell cytotoxicity in humans than the PRO treatment without the oligosaccharide (21).

The capacity of lymphocytes isolated from the spleen, MLN and PP to proliferate following mitogen activation was not affected by the dietary treatments. This conflicts with animal studies on strains of LAB which found that consuming yogurt ($\sim 1.4 \times 10^9$ bacteria/d) or pure bacteria increased the proliferative responsiveness of lymphocytes to ConA (6,16). Again, differences in the composition of the control diets may explain the differing outcomes of these studies.

The PRE treatment significantly stimulated IL-10 production by PP cells. In PP, IFN and IL-10 are primarily produced by T-helper1 lymphocytes (IFN) and by T-helper2/T-regulatory lymphocytes and dendritic cells (IL-10). The strong correlation between the level of production of the two cytokines ($r = 0.90$, $P < 0.0001$) suggests that the PRE treatment simultaneously activated different T-lymphocyte subpopulations and/or dendritic cells. The PRE treatment did not affect splenocytes or mesenteric lymphocytes, which constitutively

express lower levels of multiple cytokine transcripts than PP (22). In contrast to the strong effects of PRE on PP cytokine production, the combined treatment with PRO and PRE abrogated this effect. Because PRE did not affect the proliferative responsiveness of PP to the mitogen ConA, the increase in cytokine production indicates that PRE and/or its metabolites may interfere specifically with the regulatory processes of cytokine production. A recent study with mice also reported that consuming fructooligosaccharide enhanced IFN and IL-10 production by PP cells (23), confirming the results of the present study. Consuming the prebiotic raffinose further increased IL-12 production of PP (24), which enhances IFN production in PP (25). However, feeding mice yogurt LAB (*L. bulgaricus*, *S. thermophilus*) had no effect on basal IFN mRNA expression in PP (26). In another study, feeding mice *L. rhamnosus* for 4 wk significantly increased IFN production by splenocytes, but *B. lactis* had no effect (18). Because we used both LAB strains in combination, this may have impeded enhanced IFN production by splenocytes in the present study.

Treatment with SYN (ileum) and PRE (cecum) increased concentrations of total sIgA in rats, whereas PRO alone had no effect. The availability of PRE in the ileum may have supported the growth of the supplemented PRO and consequently stimulated sIgA synthesis. In contrast, in the cecum there is a large quantity of endogenous microorganisms; PRE supported the growth of these bacteria, which also stimulated sIgA production. However, in rats treated with SYN, the PRE may have been metabolized in the ileum and therefore could not further support bacterial growth in the cecum. Our results are in line with those of a recent study that found that feeding mice fructooligosaccharides increased fecal IgA concentration (23). The mechanism of this sIgA-enhancing effect is unknown. The IFN stimulates expression of the secretory component for IgA by epithelial cells (27). However, we found no correlation between changes in IFN production by PP and caecal sIgA concentration with the PRE treatment (data not shown). This argues against a potential role of IFN.

In conclusion, PBMC are sensitive ($CD4^+$, oxidative burst, NK) to PRO and SYN treatment, whereas PP are primarily sensitive to PRE treatment (cytokine and sIgA production). The spleen and MLN were not affected by any of the treatments. The present data suggest that PRO and PRE act via different mechanisms. Because we used a high fat, low fiber diet rather than the standard low fat rat diet used by most other studies, our results may be more relevant to the situation of humans consuming PRO or PRE in Western-style diets.

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Intestinal immunity of rats with colon cancer is modulated by oligofructose-enriched inulin combined with *Lactobacillus rhamnosus* and *Bifidobacterium lactis*

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ABSTRACT

Probiotics (PRO) are known to modulate immunity in animals and humans and to inhibit colon carcinogenesis in experimental models, while the effect of prebiotics (PRE) and synbiotics (SYN) are not well studied yet. Therefore, the effects of PRO (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12), PRE (inulin-based enriched with oligofructose, 10 % w/w), and SYN (combination of PRO and PRE) on the immune system of rats were investigated in the azoxymethane (AOM)-induced colon cancer model. After 31 weeks, rats with and without AOM-treatment were sacrificed and immune cells were isolated from spleen, mesenterial lymph nodes (MLN), and Peyer's patches (PP). AOM-treatment significantly reduced NK cell cytotoxicity (spleen, PP) of control and PRO-supplemented rats but not in rats supplemented with SYN or PRE (spleen). In addition, SYN supplementation increased NK cell cytotoxicity in PP of AOM-treated rats compared to control rats ($p < 0.01$). SYN and PRE supplementation stimulated interleukin-10 production in PP of these rats ($p<0.01$) and in MLN of rats not treated with AOM ($p < 0.05$). Interferon-γ production in PP was decreased by PRO supplementation (PRO and SYN groups combined; $p < 0.05$). Proliferative responsiveness of lymphocytes (PP) of AOM-treated rats was suppressed in SYN supplemented rats ($p < 0.01$). Overall, PRE and SYN supplementation of carcinogen-treated rats primarily modulated immune functions in the PP which coincided with a reduced number of colon tumors. PRE and SYN may contribute to the suppression of colon carcinogenesis by modulating the gut-associated lymphoid tissue.

Key words:
Immune sytem, colon cancer, probiotic, prebiotic, synbiotic, rat

Abbreviations used:

AOM = azoxymethane, ConA = concanavalin A, GALT = gut-associated lymphoid tissue, IFN = interferon-γ, IL = interleukin, LAB = lactic acid bacteria, MLN = mesenteric lymph nodes, NK = natural killer, PP = Peyer's patches, PRE = prebiotic, PRO = probiotics, SCFA = short-chain fatty acids, SYN = synbiotic

Introduction

Diet plays an important role in cancer prevention by modifying the activity of a number of protective systems including the immune system. Among the different immune cell types involved in recognition of tumor cells are the natural killer (NK) cells. Through their production of immunoregulatory cytokines and their cytotoxic effects NK cells are primary effector cells in tumor cell elimination (Smyth *et al.* 2001). They comprise up to 15% of peripheral blood lymphocytes and occur in liver, spleen, intestinal tissue, and lymph nodes. Depletion of NK cells in vivo leads to enhanced tumor formation in several mouse tumor models (Smyth *et al.* 2001). In addition, cancer patients who had recurrences within two years of surgery had significantly lower preoperative NK cell cytotoxicity than recurrence-free patients (Tartter *et al.* 1987). Data from a prospective cohort study indicate that in healthy humans a low NK cell cytotoxic activity is associated with an enhanced risk of epithelial cancer development in later life (Imai *et al.* 2000). These data suggest that a high NK cell cytotoxicity is associated with lower cancer rates.

Increasing evidence from experimental and human studies suggests that probiotics (PRO) modulate the host resistance against intestinal infections as well as a number of immune cell functions (Cross, 2002; Teitelbaum & Walker, 2002). Several studies in animals and humans have shown that PRO specifically enhance NK cell cytotoxicity in blood and spleen (Gill *et al.* 2000; Gill *et al.* 2001; Gill & Cross, 2002). Enhanced NK cell cytotoxicity through supplementation with *Lactobacillus casei* Shirota was associated with delayed carcinogenesis in a 3-methylcholanthrene-induced carcinogenesis model (Takagi *et al.* 2001). In NK-deficient mice, however, it failed to suppress tumorigenesis (Takagi *et al.* 2001).

Besides PRO, prebiotics (PRE) are another potentially cancer-protective food constituent. PRE are non-digestible carbohydrates that selectively stimulate the growth of bacteria in the colon (van Loo *et al.* 1999). Several studies observed reduced tumor numbers in the colon of rats supplemented with PRE (Pool-Zobel *et al.* 2002; Verghese *et al.* 2002).

We have recently shown that the administration of PRE alone or in combination with PRO reduced the number of colon tumors in rats treated with the carcinogen azoxymethane (AOM) (Femia *et al.* 2002).

At present, the effects of PRE on the immune system have so far only been investigated in a few studies (Schley & Field, 2002). Studies in animals using oligofructose reported increased leukocyte and/or lymphocyte numbers in the gut-associated lymphoid tissue (GALT) as well as elevated numbers of Peyer's patches (PP) (Pierre *et al.* 1997). In a short-term study we have shown that at the level of the GALT PRE as well as the combined administration of PRE and PRO (synbiotic, SYN) significantly stimulated secretory IgA and interleukin (IL)-10 production (Roller *et al.* 2004).

The present study investigated the long-term effects of PRO, PRE and SYN on the immune system of rats focusing primarily on the GALT. We hypothesized that rats exposed to the colon carcinogen AOM and treated with PRO/PRE alone or in combination would differ in their immune responses. Cytotoxicity of NK cells was assessed with cells isolated from PP, mesenteric lymph nodes (MLN), and spleen. All immunological measurements were done 31 weeks after the AOM-treatment, a time-point when the AOM-treated rats had developed adenomas and carcinomas in the colon.

Materials and methods

Materials

AOM was purchased from Sigma (Milan, Italy). Dietary components were purchased from Piccioni (Gessate, Milan, Italy). Raftilose Synergy1® was provided by Orafti (Tienen, Belgium). This PRE is an oligofructose enriched inulin which is a 1:1 mixture of long-chain and short-chain fractions of inulin, a β(2-1)-fructan extracted from chicory roots (*Cichorium intybus*). The probiotics *L. rhamnosus* GG (LGG) and *B. lactis* Bb12 (Bb12) were provided by Valio (Helsinki, Finland) and purchased from Chr. Hansen (Horsholm, Denmark),

respectively. They were provided as freeze-dried powder in sealed packets which contained ~$4x10^{11}$ cfu/g (LGG) and ~$3x10^{10}$ cfu/g (Bb12). The bacteria were kept at –20°C until used. The concentration of LGG and Bb12 in the diet was evaluated as described elsewhere (13).

Animals and treatments

Male Fischer 344 rats (Nossan, Correzzana, Milan, Italy) aged 4-5 weeks were used for this study. The experimental protocol was approved by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy. Details of the treatment were described earlier (Femia *et al.* 2002). Briefly, rats were randomly allocated to the following experimental groups: (a) controls (n = 32) were fed with a high-fat diet (HF) based on the AIN76 diet (1977), modified to contain a high amount of fat (230 g corn oil/kg diet) and a low level of cellulose (20 g/kg) as the diet typical of western human populations at high risk of colon cancer (Femia *et al.* 2002). The source of carbohydrates in this diet was sucrose (361 g/kg) and maltodextrins (100 g/kg). (b) PRO group (n = 32): rats were fed with the same HF diet as controls but supplemented with LGG (5.0±1.3x10^8 cfu/g diet) and Bb12 (5.5±2.4x10^8 cfu/g diet). (c) PRE group (n = 33): rats were fed with the same HF diet as controls but maltodextrins were replaced by 100 g/kg of Raftilose Synergy1®. (d) SYN group (n = 32): rats were fed with the same HF diet as the PRE group supplemented with LGG and Bb12 to provide ~ 5x10^8 cfu of each strain/g diet as in the PRO group. Food and water were offered ad libitum. Diets were prepared every 2 weeks, divided into aliquots and frozen at -20°C. Ten days after starting to feed the experimental diets, all rats were administered AOM (15 mg/kg two times s.c., 1 week apart) except 4-5 animals in each dietary group which were treated with saline instead of AOM and served as control for the carcinogen treatment. After a feeding period of 33 weeks with the experimental diets, the rats were sacrificed by CO_2 asphyxiation. While for the assessment of tumor incidence all the 32-33 rats/group exposed to AOM were investigated, for the immunological investigations 15 AOM-exposed rats randomly chosen from each group were used. After rats were killed, spleens, MLN and PP

were immediately send (at 4°C) from Florence to Karlsruhe by courier so that tissues were processed within 24 hr from the sacrifice.

Preparation of immune cell suspensions

For the preparation of cell suspensions from spleens, MLN, and PP RPMI-1640 culture medium containing 50 mL/L heat-inactivated fetal bovine serum, L-glutamine (2 mmol/L), penicillin ($1x10^5$ U/L), streptomycin (100 mg/L), and HEPES (25 mmol/L) was used (all components were purchased from Life Sciences, Eggenstein-Leopoldshafen, Germany). Technical details of the cell isolation procedure have been described earlier (Watzl *et al.* 1999).

Fluorescence staining of lymphocyte subpopulations

The expression of cell surface markers on the immune cells of spleens and MLN was investigated by immunofluorescence as described earlier (Watzl *et al.* 1999). Briefly, 100 µL of cell suspensions ($1x10^{10}$ cells/L) were incubated with 5 µL phycoerythrin-conjugated mouse anti-rat monoclonal antibodies to CD4 (Caltag, Hamburg, Germany) and fluorescein-conjugated mouse anti-rat monoclonal antibodies to CD8 (Caltag). In the control tube cells were incubated with appropriate isotype controls (Caltag). Analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Cytotoxicity of natural killer (NK) cells

A flow cytometric method was used to assess NK cell activity of immune cells of spleen, MLN and PP. Cells from the mouse Moloney leukaemia cell line, YAC-1, were used as target cells at the effector:target ratio of 12.5:1 and 6.25:1. Details of the assay have been described earlier (Watzl *et al.* 2000)

Lymphocyte proliferation

Ex vivo proliferative responsiveness of lymphocytes isolated from spleen, MLN (each 5×10^9 cells/L) and PP (7×10^9 cells/L) to the mitogen ConA (1 mg/L, Sigma-Aldrich) for 72 h at 37°C, 5 % CO_2 and 95 % humidity was determined by ELISA (Cellular Proliferation

ELISA kit, Roche Diagnostics, Mannheim, Germany). Proliferative responses were expressed as the net absorbance values (absorbance$_{A450nm-A650nm}$ of pulse-labeled cells - absorbance $_{A450nm-A650nm}$ of unlabeled cells) and expressed as optical density (OD) units.

Cytokines

Splenocytes (100 µL; 1 x 10^9 cells /L for IFN-γ and 5 x 10^9 cells/L for IL-10), MLN (100 µL; 1 x 10^9 cells /L for IFN-γ and 5 x 10^9 cells/L for IL-10) and PP (100 µL; 5 x 10^9 cells/L for IFN-γ and IL-10) were cultivated in duplicate in flat-bottomed 96-well microtiter plates and stimulated by 100 µL of Con A (5 mg/L) for 24 h at 37°C, 5 % CO_2 and 95 % humidity. IFN-γ and IL-10 in supernatants were measured by commercial ELISA kits OptEIA™ (BD Pharmingen, Heidelberg, Germany) following the manufacturers' instructions.

Statistical evaluation of the data

Results are reported as means ± standard deviation (SD). Differences between control and treatment groups were tested for significance by ANOVA with the Tukey-Kramer test for comparison of individual means when appropriate. Differences between AOM-treated and non-treated animals per group were analyzed by using unpaired Student's t-test. To assess correlations between immunological markers and numbers of tumors, Pearson correlation coefficients were computed. Values of $p < 0.05$ were considered significant. All statistical calculations were performed with the StatView program (SAS Institute 1998, Cary, NC, USA).

Results

Body weight and feed intake

Supplementation of the control diet with PRO, PRE and the combined application of PRO and PRE (SYN) for 33 weeks had no significant effect on feed intake and weight gain when compared with the control group (data not shown). The mean weight of the rats at the

beginning of the experiment was 108.8 ± 12.2 g (n = 129). At death, the mean weight was similar among dietary groups (479.5 ± 34.0).

Tumour suppressive effect of dietary treatment

We already reported that AOM-treated rats receiving Raftilose Synergy1 (PRE and SYN groups) had a significantly lower number of tumors (adenomas and carcinomas; $p < 0.001$) than rats without the prebiotic supplement (control and PRO) (Femia *et al.* 2002). The probiotic treatment (PRO and SYN groups) slightly reduced the number of malignant tumours ($p = 0.079$) when compared to control and PRE groups.

NK cell activity

NK cell-like activity was measured in spleens, MLN and PP. In control and PRO-supplemented rats the AOM-treatment reduced NK-cell like activity in all tissues investigated (Table 1). PRE (PP) and SYN (PP and spleen) treatment prevented the AOM/tumor-associated suppression of NK cell cytotoxicity measurable in control and PRO-fed rats. PP of SYN-supplemented rats treated with AOM showed significantly higher NK cell activity compared with controls or PRO fed rats (Table 1). In addition, in the spleen of rats not exposed to the carcinogen supplementation with PRE also stimulated NK cell cytotoxic function. However, no significant correlation was observed between the total number of adenoma or carcinoma in the colon and the cytotoxic activity of NK cells independent of the dietary group (data not shown).

Cytokine production

IFN and IL-10 production of ex vivo activated cells isolated from spleen, MLN, and PP were measured. IL-10 production in PP of AOM-treated rats was significantly enhanced in rats supplemented with PRE and SYN (Table 2). This stimulatory effect on IL-10 production was also seen in the MLN of rats not treated with the carcinogen but supplemented with the prebiotic (PRE and SYN combined vs. control and PRO combined, 141.9±38.8 ng/L vs.

76.3±20.3 ng/L, $p < 0.05$). IL-10 production by splenocytes was neither affected by dietary supplements nor by AOM-treatment. In contrast, the capacity to produce IFN was only affected by the dietary treatment in the PP of rats not exposed to AOM (data not shown). Rats supplemented with the probiotics (PRO and SYN) showed significantly lower IFN production when compared with control and PRE rats (1.4±0.5 µg/L vs. 2.2±0.7 µg/L; $p < 0.05$). No significant effects were observed in spleen and MLN.

Lymphocyte subpopulations

Spleen and MLN lymphocytes were analyzed for the percentage of $CD4^+$ T-helper and $CD8^+$ T-suppressor lymphocytes. AOM-treatment significantly reduced the ratio of CD4:CD8 lymphocytes in spleen and MLN (Table 3). In both tissues this was caused by an increase in the number of CD8-positive lymphocytes ($p < 0.05$; data not shown). In rats without AOM-treatment PRE reduced the CD4:CD8 ratio in spleens (Table 3) without significantly changing the percentage of CD4 or CD8 (data not shown). Cells isolated from PP were not available for the determination of the lymphocyte subpopulations.

Lymphocyte proliferation

The treatment with the carcinogen did not affect lymphocyte proliferation compared to untreated rats. In AOM-treated rats no significant effects of the dietary intervention were observed in spleens and MLN (data not shown). In PP, however, the SYN treatment significantly reduced lymphocyte proliferation (control 1.4±0.5 OD_{450}, PRO 1.2±0.3 OD_{450}, PRE 1.3±0.4 OD_{450}, SYN 0.6±0.4 OD_{450}, $p < 0.01$ vs. control, PRO and PRE).

DISCUSSION

The results of the present study demonstrate that long-term supplementation with PRE and SYN modulates immune functions in the GALT, while PRO alone had only minor effects. Rats supplemented with PRE or SYN developed less tumors than control rats and showed increased NK cell cytotoxic function in the PP.

In rats not exposed to the carcinogen the PRE supplementation significantly increased NK cell activity of splenocytes. Only few studies so far have investigated the effects of PRE on the immune system. In mice supplementation with the non-digestible oligosaccharides nigerooligosaccharides stimulated hepatic NK cell cytotoxicity (Murosaki *et al.* 2002), while inulin and oligofructose enhanced splenic NK cell function (Kelly-Quagliana *et al.* 2003) confirming the results of the present study. In vitro, nigerooligosaccharides also stimulated NK cell function pointing to a direct effect of such oligosaccharides on NK cells via specific lectin-type receptors (Murosaki *et al.* 1999).

The AOM-treatment itself significantly suppressed NK cytotoxicity in spleens and PP of control and PRO-/PRE-supplemented rats. This suppression was prevented in the rats supplemented with SYN (spleens) and with SYN and PRE (PP). Although several studies have demonstrated that AOM-treatment inhibited NK cell cytotoxic activity of splenocytes (Baten *et al.* 1989; Altmann & Lala, 1994; Sekine *et al.* 1997; Exon & South, 2003), our study is the first to show that NK cell cytotoxicity is also suppressed by AOM in cells isolated from the PP. Whether this suppression of NK cells in PP is involved in colon carcinogenesis is currently not known. However, the probiotic *Lactobacillus casei* Shirota enhanced cytotoxicity of NK cells and delayed tumor onset in mice with normal NK cell functions, while in a NK cell-deficient mouse mutant it was not effective (Takagi *et al.* 2001). This suggests that not only the suppression of NK cell functions in spleens but also in PP may contribute to tumor growth. In the *Min* mouse model supplementation with short-chain fructo-oligosaccharides reduced the occurence of colon tumors and increased the number of PP in the GALT (Pierre *et al.* 1997). However, the cytotoxicity of NK cells in these PP was not measured.

Our data indicate that primarily the supplementation with PRE affected tumor incidence and NK cell function. In contrast to the effects of PRE, the long-term supplementation of AOM-exposed rats with the two probiotic strains had no significant effect

on NK cell function and minor effects on tumorigenesis. Several other studies with PRO reported stimulation of NK cell cytotoxicity and/or delayed colon carcinogenesis (Singh *et al.* 1997; McIntosh *et al.* 1999; Takagi *et al.* 2001). However, these studies supplied only one probiotic strain and for shorter supplementation periods as in the present study. A further point of our study is that we have used a high-fat diet comparable to a Western-type diet which at least in humans is known to suppress NK cell cytoxicity (Barone *et al.* 1989). The standard rat chow, however, is a low-fat diet. In another animal study only *L. rhamnosus* treatment significantly increased NK cell activity, while *B. lactis* had no significant effect (Gill *et al.* 2000). Since we have used both lactic acid bacteria (LAB) strains in combination, this may have impeded the enhanced NK cell cytotoxicity observed with *L. rhamnosus* alone. NK cytotoxicity of MLN cells was not affected by the dietary supplementation. After short-term supplementation also no effect was observed in this tissue (Roller *et al.* 2004) suggesting that NK cells in these lymph nodes are not responsive to this dietary treatment.

Cytokine production in the GALT was modulated by the dietary supplements. PRE and SYN supplementation significantly stimulated IL-10 production by PP cells in AOM-treated rats. Recently, $CD4^+CD25^+$ regulatory lymphocytes have been shown to require IL-10 to interrupt colon carcinogenesis in mice (Erdman *et al.* 2003). Short-term exposure to the same PRE also enhanced IL-10 production in PP (Roller *et al.* 2004). A recent study reported enhanced IL-10 production by PP cells after feeding of fructooligosaccharide to mice (Hosono *et al.* 2003) confirming the results of the present study. Feeding the non-digestible oligosaccharide raffinose to mice further resulted in increased IL-12 production of PP (Nagura *et al.* 2002) which is known to enhance IFN production in PP (MacDonald & Monteleone, 2001) as well as NK cell cytotoxicity (Trinchieri, 1995). Incubation of PP cells with raffinose in vitro did not increase IFN or IL-12 production suggesting that in vivo indirect effects of raffinose (e.g., butyrate production, altered intestinal microflora) are mediating the observed effects. In contrast to PP, cytokine secretion of splenocytes was not

modulated by the dietary treatment. In MLN, however, PRE and SYN supplements stimulated IL-10 production in untreated and AOM-treated rats.

While supplementation with PRE or SYN primarily stimulated immune functions of the GALT, probiotics supplementation inhibited IFN production by PP of rats not treated with AOM. Immune cells with the capacity to produce IFN include NK cells which occur as subtypes with high cytotoxic/low IFN-producing capacity ($CD56^{dim}$) and low cytotoxic/high IFN-producing capacity ($CD56^{bright}$) (Cooper *et al.* 2001). Since in rats (-AOM) of the present study supplemented with the two probiotic strains NK cell cytotoxicity was high, the PRO supplement may have induced a shift in the NK subtypes towards cells with a higher cytotoxic and a lower IFN-producing capacity. In another study, nigerooligosaccharides stimulated lytic function of hepatic NK cells but not IFN secretion (Murosaki *et al.* 2002). Oral exposure of mice to yoghurt LAB (*L. bulgaricus*, *S. thermophilus*), had no effect on basal IFN mRNA expression in PP (Tejada-Simon *et al.* 1999). Taken together, these results are in line with the present data. Feeding mice with *L. rhamnosus* for 4 weeks significantly increased IFN production by splenocytes, while *B. lactis* feeding had no significant effect (Gill *et al.* 2000). Since we have used both LAB strains in combination, again this may have impeded enhanced IFN production by splenocytes in our study.

AOM-treatment significantly reduced CD4:CD8 ratio in spleen and MLN primarily by increasing the percentage of $CD8^+$ lymphocytes. In spleens of rats not treated with AOM the PRE supplement further decreased the CD4:CD8 ratio. Contradictory results were recently reported demonstrating in mice that a 2-weeks feeding period with fructooligosaccharides had no influence on the CD4:CD8 ratio in PP (Manhart *et al.* 2003). In mice, feeding of LAB for 4 weeks induced no significant effects on the percentages of $CD4^+$ and $CD8^+$ cells in the blood (Gill *et al.* 2000). Altogether these data suggest that PRO and PRE have only minor effects on the composition of T cell subsets in different immune compartments.

The capacity of lymphocytes isolated from spleen and MLN to proliferate following mitogen activation was clearly not affected by the dietary treatment. However, in PP supplementation with SYN reduced lymphocyte proliferation. This is in contrast to results from animal studies with strains of LAB which showed that feeding of yoghurt (about $1.4x10^9$ bacteria/d) or of pure bacteria resulted in significantly higher proliferative responsiveness of lymphocytes to ConA (Gill *et al.* 2000; Aattouri *et al.* 2002). Again, differences in the composition of the control diets may explain the differing outcomes of these studies.

The mechanisms by which PRE and SYN act on the GALT and on tumorigenesis are not clear. Besides the direct immunological effects of PRO which have been demonstrated in a number of studies (Gill & Cross, 2002), PRE may affect the immune system by several ways. First, PRE may induce a shift in the intestinal microflora towards bifidobacteria changing the antigen pattern of the intestinal microflora (Teitelbaum & Walker, 2002). Second, we have shown that supplementing the rat feed with the prebiotic increased the production of short-chain fatty acids (SCFA) such as butyrate in the caecum (Femia *et al.* 2002). Butyrate is known to suppress lymphocyte proliferation, to inhibit cytokine production of TH1-lymphocytes, and to up-regulate IL-10 production (Säemann *et al.* 2000; Cavaglieri *et al.* 2003).

In conclusion, the reduced number of AOM-induced colon tumors in rats supplemented with PRE or SYN coincided with a marked stimulation of immune functions within the GALT. The present data clearly suggest that PP are the primary lymphoid tissue responsive to the oral intake of PRE or SYN. Whether this modulation of PP cells is associated with the suppression of carcinogenesis in the colon and the underlying mechanisms have to be determined in future studies.

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Tab. 1. NK cell activity (effector:target ratio 12.5:1) of mononuclear cells isolated from spleen, mesenteric lymph nodes or Peyer's patches from rats fed for 33 weeks with a probiotic, prebiotic, synbiotic or control diet. Rats were either non-exposed (n = 4-5 per group) or exposed to the carcinogen AOM (n = 15 per group) at the beginning of the dietary treatment. Data are expressed as percentage of lysed target cells.

AOM	Control	Probiotic	Prebiotic	Synbiotic
		Spleen		
-	31±4[1]	39±2[3]	43±10[*,4]	33±3
+	22±8	23±10	25±8	28±10
		Mesenteric lymph nodes		
-	29±5	36±6[2]	30±5	33±8
+	27±8	26±7	27±6	27±5
		Peyer's patches		
-	39±2[2]	47±4[3]	39±4	45±9
+	32±6	34±8	39±8	42±6[**]

[*] Mean differs compared to control ($P < 0.05$).

[**] Mean differs compared to control ($P < 0.01$) and probiotics ($P < 0.05$).

[1]: $p = 0.057$ vs. AOM-treated rats.

[2]: $p < 0.05$ vs. AOM-treated rats.

[3]: $p < 0.01$ vs. AOM-treated rats.

[4]: $p < 0.001$ vs. AOM-treated rats.

Tab. 2. Interleukin-10 (ng/L) production of mononuclear cells isolated from spleen, mesenteric lymph nodes and Peyer's patches from rats fed for 33 weeks with a probiotic, prebiotic, synbiotic or control diet. Rats were either non-exposed (n = 4-5 per group) or exposed to the carcinogen AOM (n = 15 per group) at the beginning of the dietary treatment.

AOM	Control	Probiotic	Prebiotic	Synbiotic
		Spleen		
-	592±180	644±201	956±480	722±138
+	687±139	748±147	677±168	646±272
		Mesenteric lymph nodes		
-	83±21[1]	69±20[1]	144±26*	140±56*
+	136±40	138±38	178±44	166±60
		Peyer's patches		
-	270±55	220±151	297±64	308±64
+	197±85	260±65	331±99**	324±147**

*: Means differ compared to probiotics ($P < 0.05$).

**: Means differ compared to control ($P < 0.01$).

[1]: $p < 0.05$ vs. AOM-treated rats.

Tab. 3. CD4+/CD8+ ratio of lymphocytes (%) isolated from spleen and mesenteric lymph nodes from rats fed for 33 weeks with a probiotic, prebiotic, synbiotic or control diet. Rats were either non-exposed (n = 4-5 per group) or exposed to the carcinogen AOM (n = 15 per group) at the beginning of the dietary treatment.

AOM	Control	Probiotic	Prebiotic	Synbiotic
		Spleen		
-	13.5±2.4[2]	10.4±2.8[2]	7.9±1.8[*]	9.7±3.3[1]
+	7.6±2.8	6.1±2.2	6.0±2.4	6.2±2.1
		Mesenteric lymph nodes		
-	8.7±2.1[2]	6.7±1.3	7.7±0.5[2]	8.0±2.0[1]
+	5.1±1.7	4.8±1.3	5.9±1.1	5.9±1.3

[*] Mean differs compared to control ($P < 0.01$).

[1]: $p < 0.05$ vs. AOM-treated rats.

[2]: $p < 0.01$ vs. AOM-treated rats.