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Carcinogen-DNA and Protein Adducts: Biomarkers for Cohort Selection and Modifiable Endpoints in Chemoprevention Trials

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Abstract Chemical-specific markers have been developed for a number of environmental carcinogens for use as molecular dosimeters of individual exposure. In addition to contributing substantially to the specificity and sensitivity of epidemiological studies aimed at determining the role of environmental agents in the etiology of human cancers, some of these biomarkers may prove to be useful endpoints for assessing the efficacy of preventive interventions, including exposure avoidance or remediation and chemoprevention. Biomarkers of the biologically effective dose may be particularly useful in this context in that they provide a mechanistic linkage between exposure and disease outcome. The biologically effective dose reflects the amount of toxicant that has interacted with its critical molecular target and can be measured through a variety of analytical techniques as either carcinogen-DNA or -protein adducts. Approaches for the development and validation of aflatoxin adduct biomarkers are presented as a paradigm for the application of carcinogen-specific markers for cohort selection and as modifiable endpoints for assessing efficacy in chemoprevention trials. J. Cell. Biochem. 25S:85–91. © 1997 Wiley-Liss, Inc.

Key words: biologically effective dose; aflatoxin-N^7-guanine; aflatoxin-albumin adducts; oltipraz; hepatocarcinogenesis

Increased understanding of the mechanistic basis of chemical carcinogenesis provides opportunities for the identification of molecular biological markers reflecting events from exposure through clinical disease. These molecular markers can be classified into three major categories: markers of exposure reflecting either internal or biologically effective dose of carcinogens, markers of effect indicating a biological response to an exposure, and markers of susceptibility that characterize the inherent sensitivity of an individual to toxins and carcinogens. While measurements of internal dose provide unequivocal identification of chemical exposure, they do not provide evidence that toxicologically relevant damage has occurred. Because the nature and extent of toxicological responses are determined by the interaction of toxicants with their molecular targets, assessment of the biologically effective dose indicates that initiation of the toxicological process has occurred. Application of these biomarkers to populations improves the accuracy of exposure measurements and leads to the identification of susceptible individuals in the presence of adverse exposures. The selection of susceptible or "at-risk" individuals for participation in intervention studies, be they exposure abatement or chemoprevention trials, provides for a more homogeneous study population with reduced requirements for sample size.

Although markers of the biologically effective dose are typically sophisticated surrogates of exposures, it has been possible in some instances to establish a linkage between these marker measurements and risk of disease. The validation of any biomarker-disease link requires a series of studies in experimental animals and humans. Ideally, an appropriate animal model is used to determine the associative or causal role of the marker in the disease

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pathway and to establish relationships between dose and response. The putative marker can then be validated in pilot human studies where sensitivity, specificity, accuracy and reliability parameters are established. Data obtained in these studies can be used to assess intra- or inter-individual variability, background levels, and relationship of the biomarker to external dose or to disease outcome, as well as feasibility for use in larger population-based studies. It is important to establish a connection in humans between the marker and the exposure and/or the outcome of interest. To fully interpret the information that a marker can and cannot provide, prospective epidemiologic studies may be necessary to demonstrate the role that the marker plays in the overall carcinogenic process. Once a biomarker-disease link has been established it becomes possible to use these biomarkers as short-term endpoints in chemoprevention trials. The successful implementation of this approach requires that the interventions act on the early, toxicokinetic events associated with carcinogen exposures.

A number of recent books and reviews discuss the utilization of biomarkers in many aspects of epidemiology and toxicology [1–5]. This article highlights the approaches taken for the development, validation and application of aflatoxin-DNA and -protein adduct biomarkers to cancer prevention studies. The aflatoxins are potent hepatocarcinogens and hepatotoxins in experimental animals and are classified as Group I known human carcinogens by the International Agency for Research on Cancer [6]. To date, aflatoxins are among the few carcinogens for which rigorous biomarker development has been undertaken. The general approach for the validation of aflatoxin biomarkers prior to their application to cancer prevention trials is outlined in Figure 1.

METHODS FOR MEASURING AFLATOXIN BIOMARKERS

The classification of aflatoxins as a definitive human carcinogen has led to a need for accurately relating exposure to these mycotoxins to risk of developing disease. Among the various possible biomarkers of aflatoxin exposure, the measurement of carcinogen-DNA and protein adducts are of major interest because they are direct products of or surrogate markers for damage to a critical macromolecular target and are derived from the ultimate carcinogenic species exo-aflatoxin-8,9-oxide. While it is not possible to routinely assay aflatoxin-DNA adduct levels in humans, the major DNA adduct species formed in vivo, aflatoxin-N^7-guanine, is rapidly excised and eliminated as a modified guanine base. Urine appears to be the sole route of elimination of this carcinogen-DNA adduct. Serum albumin is the predominant blood protein to be alkylated following exposure to aflatoxin. The aflatoxin-lysine adducts in albumin do not appear to be repaired; the biological half-life for circulating albumin is 2–3 weeks. Based upon these kinetics, it is assumed that measurements of aflatoxin-DNA adducts in urine reflect recent exposures while levels of aflatoxin-albumin adducts in serum reflect cumulative, multiple exposures.

Measurements of the aflatoxin DNA and protein adducts ideally require techniques that are sensitive, specific and amenable to large numbers of samples. Methods that have been employed include chromatographic methods such as thin layer and HPLC, immunological assays using specific antibodies or antisera such as enzyme-linked immunosorbent assays, radioimmunoassays and immunochemical visualization in tissues, and instrumentation-based methods such as synchronous fluorescence spectroscopy. Each of these methodologies has its own characteristics for sensitivity and specificity which must be considered in the context of the application. We have taken advantage of the inherent strengths of both antibody selectivity and chromatographic separations to develop an immunoaffinity chromatography/HPLC procedure to isolate and sensitively measure aflatoxins in urine samples [7]. However, from a practical perspective pertinent to chemoprevention trials, measurement of aflatoxin-albumin adducts in serum by immunoassay offers the most reasonable approach for studies with large numbers of people or serial sampling [8].

RELATIONSHIPS OF AFLATOXIN BIOMARKERS TO EXPOSURE: EXPERIMENTAL AND EPIDEMIOLOGICAL STUDIES

Studies by Bennett et al. [9] and Groopman et al. [10] demonstrated dose-dependent increases in the levels of aflatoxin-N^7-guanine in urine following acute treatment of rats with aflatoxin B_1. Moreover, striking linear correspondence between the amounts of aflatoxin-N^7-guanine excreted in urine over the initial 24 post-dosing period and residual levels of he-

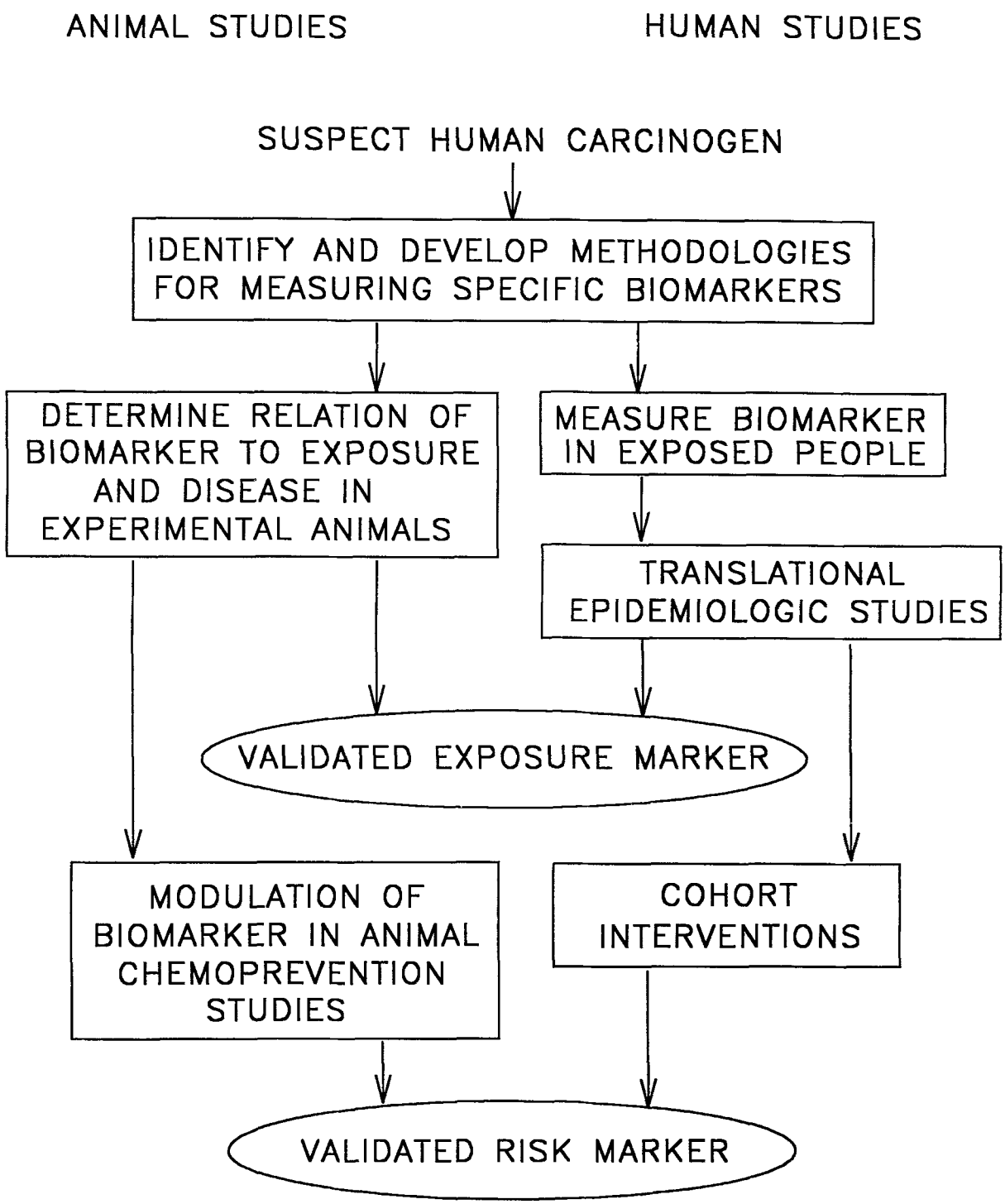

Fig. 1. Paradigm for the development, validation and application of carcinogen-DNA and protein adducts to cancer prevention studies.

patic aflatoxin-DNA adducts were observed in both studies. Excretion patterns in rat urine of several other oxidative metabolites of aflatoxin were not dose-dependent. The relationship between hepatic and urinary levels of aflatoxin-DNA adducts following a single dose reflects the short biological half-life ($t_{1/2}$ = 8 h) of aflatoxin-N^7-guanine adducts in DNA. Chronic dosing of rats with aflatoxin B_1 leads to sustained excretion of aflatoxin-N^7-guanine; however, due

to cumulative patterns of aflatoxin-DNA adduct formation and changing patterns of metabolism resulting from carcinogen-mediated inductions of some phase 1 and 2 enzymes, correspondence between levels of hepatic DNA adducts and the excreted biomarker becomes more qualitative than quantitative [11]. Hepatic DNA adduct levels tend to increase with subsequent exposures while amounts of aflatoxin-N^7-guanine tend to drop during sequential 24-h collections. Strong concordance between aflatoxin B_1 exposure and serum levels of aflatoxin-albumin adducts has also been observed following acute and chronic exposures to aflatoxin B_1 [12,13]. Because of the longer biological half-life of the aflatoxin-albumin adduct biomarker in rats (2–3 days) and humans (2–3 weeks), it is likely to be more useful than urinary markers in chronic exposure settings.

Studies conducted in Guangxi Autonomous Region, an area of the People's Republic of China with high incidence of liver cancer, determined both dietary intake of aflatoxin and levels of biomarkers over a one week period [14]. Exposure resulted principally from consumption of contaminated corn. Aflatoxin B_1, aflatoxin P_1, aflatoxin M_1 and aflatoxin-N^7-guanine were routinely detected in urine samples by immunoaffinity/HPLC analyses. However, only urinary levels of aflatoxin-N^7-guanine and aflatoxin M_1 showed a dose-dependent relationship towards aflatoxin intake. Using the same study population, Gan et al. [15] monitored levels of aflatoxin-serum albumin adducts and observed a highly significant association between adduct levels and aflatoxin intake. Moreover, when the data for DNA adduct excretion in urine and serum albumin were compared, a significant relationship was seen, with a correlation coefficient of 0.73. Similar studies examining the relationship between the urinary and serum biomarkers have been conducted in The Gambia, West Africa; strong associations between dietary exposure and aflatoxin adduct levels were also observed [16,17]. Thus, the utility of these dosimetry markers as effective monitors of exposure to aflatoxins has been confirmed in two populations at high risk for liver cancer.

RELATIONSHIPS OF AFLATOXIN BIOMARKERS TO RISK: PROSPECTIVE EPIDEMIOLOGICAL STUDIES

Many ecological and case-control studies have been conducted to explore the link between aflatoxin and liver cancer through monitoring levels of aflatoxins in the diet and cancer incidence in various geographical regions of the world. While many [18], but not all, [19] of such studies indicated a positive association between aflatoxin ingestion and risk of liver cancer, most of these investigations suffered from a lack of good data on aflatoxin exposure and/or from poor information on cancer incidence. In general, the most rigorous test of an association between an agent and disease outcome is found in prospective epidemiological studies, where healthy people are monitored until the diagnosis of the disease. A nested, case-control study initiated in Shanghai in 1986 is examining the relationship between markers for aflatoxins and hepatitis B virus and liver cancer [20,21]. In this study, 18,244 urine samples were collected from healthy men between the ages of 45 and 64 years. In the subsequent years, 50 of these individuals developed liver cancer. The urine samples for these 50 cases were age and residence matched with 267 controls and analyzed for aflatoxin biomarker and hepatitis B virus surface antigen status. The data revealed a highly significant increase in the relative risk (RR=3.4) for those liver cancer cases where urinary aflatoxins were detected. The relative risk for people who tested positive for the hepatitis B virus surface antigen was about 7, but individuals positive for both urinary aflatoxins and hepatitis B virus surface antigen had a relative risk for developing liver cancer of 59. When individual aflatoxin metabolites were stratified for liver cancer outcome, the presence of aflatoxin-N^7-guanine in urine resulted in a 2–3-fold elevation in risk of developing liver cancer [21]. These studies, which provided the first demonstration of a multiplicative interaction between two major risk factors for liver cancer, i.e., hepatitis B virus and aflatoxin exposure, clearly define a relationship between presence of a carcinogen specific biomarker and cancer risk.

MODULATION OF AFLATOXIN BIOMARKERS AND RISK DURING EXPERIMENTAL CHEMOPREVENTION

It is possible to modify risk for hepatocarcinogenesis induced by aflatoxin in animals using chemopreventive interventions with phenolic antioxidants, 1,2-dithiole-3-thiones and other agents when they are administered simultaneously with the carcinogen [22]. Because risk

can be attenuated while aflatoxin B_1 exposure is held constant, these animal models are useful experimental systems for examining the relationships between aflatoxin biomarkers in biological fluids and cancer risk. Oltipraz (4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione) is a particularly effective inhibitor of aflatoxin hepatocarcinogenesis in the male F344 rat when fed beginning one week prior to and throughout carcinogen exposure [23]. In this chemoprevention model companion molecular dosimetry studies indicated that levels of aflatoxin-N^7-guanine adducts in the livers of rats fed oltipraz were reduced 65% at 24 h after dosing. While elimination of total urinary aflatoxins was indistinguishable between the control and treated groups, oltipraz pretreatment led to a 67% reduction in the urinary elimination of aflatoxin-N^7-guanine over the initial 24 h post-dosing period. Subsequent molecular dosimetry studies using repetitive aflatoxin B_1 exposure regimens coupled with chemopreventive interventions with oltipraz or its unsubstituted congener, 1,2-dithiole-3-thione, also demonstrated concordant reductions in the levels of hepatic aflatoxin-DNA adducts and the urinary and serum aflatoxin biomarkers over the carcinogen exposure period [11,24]. However, in these studies the magnitude of the reductions in levels of tissue DNA adducts and the biologically effective dose biomarkers tended to underestimate the degree of tumor inhibition by chemoprevention. This outcome is not surprising given the multicomponent nature of the carcinogenic process. Events not mediated by DNA adduct formation, such as recurrent cytotoxicity, also contribute to the risk of cancer outcome.

Although experimental cancer chemoprevention models typically employ daily interventions throughout the time of carcinogen exposure, it has been demonstrated recently that delayed, transient as well as intermittent interventions with oltipraz effectively inhibit aflatoxin-mediated tumorigenesis [25,26]. These models are more relevant to clinical opportunities for chemoprevention and provide additional means to test the association between modulation of risk and biomarkers. Egner et al. [24] examined the kinetics of aflatoxin-albumin adduct formation and removal in blood of rats undergoing a delayed, transient intervention. Oltipraz was fed for two weeks beginning one week after aflatoxin B_1 dosing began and ending two weeks before aflatoxin B_1 dosing ended. No statistically significant reduction in aflatoxin-albumin adducts levels were seen until approximately three albumin half-lives passed. However, once achieved, significant differences from the control group were maintained throughout the remainder of the aflatoxin exposure period. Collectively, these coupled chemoprevention/biomarker studies indicate that

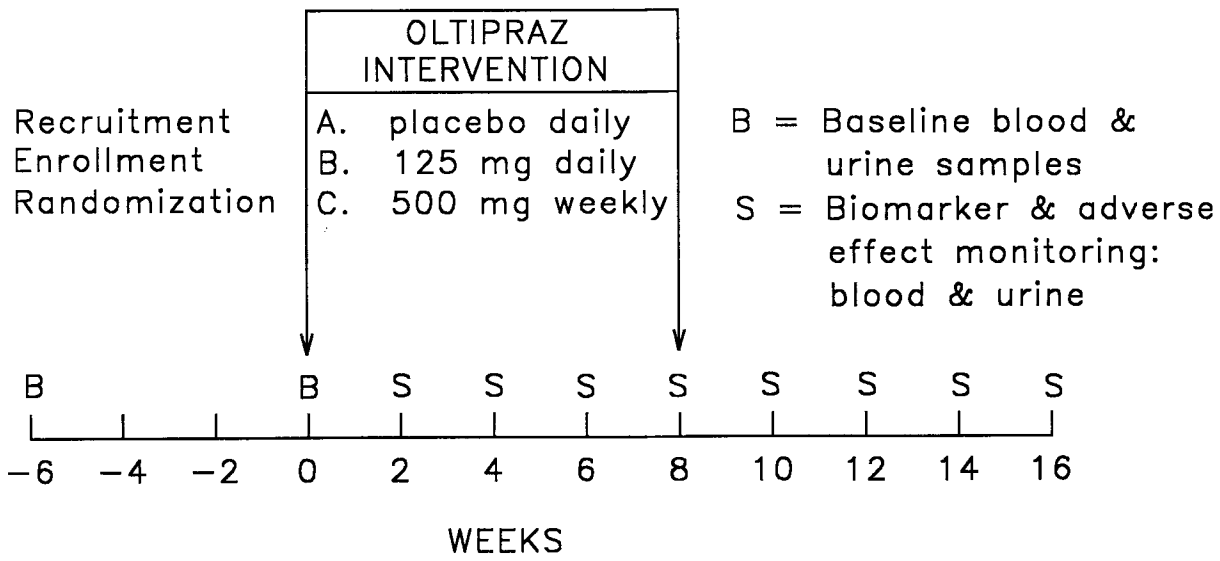

Fig. 2. General design for the oltipraz intervention trial in Qidong, People's Republic of China. Measurements of changes in the levels of aflatoxin-N^7-guanine in urine and aflatoxin-albumin adducts in serum are the biomarker endpoints used to assess possible efficacy of oltipraz in a cohort exposed to dietary aflatoxins and at high risk for the development of hepatocellular carcinoma.

measurements of aflatoxin biomarkers reflect the altered risk for disease of the protected animals.

APPLICATION OF AFLATOXIN BIOMARKERS TO IDENTIFY COHORTS AT RISK AND AS ENDPOINTS FOR INTERVENTION STUDIES

Hepatocellular carcinoma is the leading cause of cancer death in Qidong, Jiangsu Province, People's Republic of China and accounts for up to 10% of all adult deaths in some of the rural townships in this region [27]. Aflatoxins are consistent contaminants of the food supply; in a 1993 longitudinal survey of 120 residents of Daxin Township, Qidong, which lies in the hot, humid delta country of the Yangtze River, greater than 95% of the participants tested positive for serum aflatoxin albumin adducts throughout a 3 month period [28]. Moreover, characterization of the mutational spectra in the p53 tumor suppressor gene in hepatocellular carcinoma from Qidong demonstrated a high frequency (>50%) of AG*G*→AG*T* transversion mutations on the noncoding strand at codon 249 [29]. These mutations are not observed in liver cancers from low aflatoxin exposure regions of China. GC→TA transversions are the most common base substitutions produced by aflatoxins in experimental systems. As a result of the documented exposure to aflatoxins coupled with a very high risk for liver cancer, a Phase II clinical chemoprevention trial with oltipraz was conducted in residents of Daxin during the summer and fall of 1995. Over 1000 individuals were screened and 234 healthy participants (145 women and 89 men aged 25–65) were enrolled into the trial. One of the eligibility criteria was positivity for serum aflatoxin-albumin adducts; levels in the trial group ranged between 1.3 and 10 pmol/mg albumin. [30] As shown in Figure 2, this 8 week, randomized, placebo-controlled trial examined the effects of daily (125 mg) and weekly (500 mg) doses of oltipraz on levels of two aflatoxin biomarkers: aflatoxin N^7-guanine adducts excreted into urine and aflatoxin-albumin adducts in serum. Blood and urine samples were collected biweekly throughout the intervention and during a 2-month follow-up period. With approximately 80 participants in each of the 3 arms, the clinical trial has the power to determine small decreases in the levels of the urinary and/or serum aflatoxin biomarkers. It is anticipated that biomarker analyses will be completed in late 1996.

The availability of well-characterized intermediate markers reflecting the modulation of the biologically effective dose of environmental carcinogens as study endpoints allows the design and conduct of short, efficient clinical prevention trials. Study size requirements are minimized by preselection of study participants expressing the modifiable endpoint(s) of interest, while study duration can be shortened by the use of risk biomarkers that can be rapidly modulated. Hopefully, results from such trials with oltipraz will provide insights into the means to achieve large-scale reductions in the incidence of hepatocellular carcinoma in populations at high-risk for unavoidable exposures to aflatoxins. Further, such studies may serve as templates for preventive interventions targeting individuals at high risk for other environmentally induced diseases.

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