



IMARS Highlights

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What's cooking? Notes from the Editor's pot

Your editor is on vacation right now so that this message will be brief. However, this issue of Highlights again emphasizes some recurring themes. One is the potential of Maillard reaction products to contribute to a wide spectrum of disease, including neurological disorders and cancer, independent of hyperglycemia. Another is the need for careful design and interpretation of experiments relating to the effects of dietary AGEs on human health, and the role of the receptor for advanced glycation end products (RAGE) in inflammation and disease. Finally, Toshio Miyata has provided some helpful recommendations relating renal function to measurement of tissue and circulating AGEs.

For the first time our online journal contains a Letter to the editor with additional commentary on one of the articles discussed in the previous issue of *IMARS Highlights*. Hopefully it is the first of many Letters, and we encourage IMARS members to send their thoughts and comments on any present or past articles in the journal.

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Heated diets, AGEs and tissue dysfunction

By Timo Buetler

In recent years several articles have presented data on human intervention studies with excessively heated diets containing significant amounts of AGEs resulting in adverse health effects (1, 2). Recently, a new study by Stirban *et al.* (3) was added to this list of human dietary AGE intervention studies. Thirteen type II diabetic adults received a high AGE test meal after a 3-day pre-treatment with either benfotiamine or placebo. The results seem to suggest that the AGEs of the thermally treated meal induced micro- and macrovascular dysfunction as well as increased oxidative stress. These effects were blunted or blocked by benfotiamine pretreatment. While there is little doubt that the heated meals indeed had negative effects on health, to attribute these effects to AGEs is premature and several points need to be considered when interpreting this and similar other studies.

First of all, the extreme thermal treatment of the meals (20 minutes of frying/ broiling at 230°C) very likely resulted in multiple structural and compositional modifications besides AGE formation, including destruction of vitamins and antioxidants as well as generation of lipid peroxides and lipid degeneration products. With respect to thermal destruction of vitamins, Peter O'Brian has reported at the 2006 COST/IMARS meeting that even lesser thermal treatment of food (30 minutes at 121°C) was sufficient to destroy the B-vitamin thiamin. Hence it can be assumed that the test meal used in the Stirban study (3) was thiamin deficient. Thiamin is an essential co-factor of several glycolytic enzymes and thiamin deficiency results in impaired glycolysis, dicarbonyl formation and increased oxidative stress (4). Oxidative stress influences inflammation and endothelial function that can result in the observed micro- and macrovascular dysfunction. Benfotiamine is a water-soluble form of thiamin and can rescue thiamin deficiency, counteract glucose toxicity, oxidative stress and tissue damage in diabetic animals (5). Thus, the effects observed by Stirban *et al.* (3) can also be explained by thermal destruction of thiamin and its reversal by benfotiamine. This may have nothing to do with AGEs as Wu and Ren (6) recently reported that benfotiamine alleviated cerebral oxidative damage in streptozotocin diabetic mice independent of AGEs.

Even the most recent report by Cai *et al.* (7) cannot be used to argue that AGEs are the culprit for the low oxidative stress and life-extending effects observed in mice fed life-long a low AGE diet because the composition of the two diets was not identical. It is especially interesting to note that the high AGE diet used contained significantly higher levels of several vitamins and minerals compared to the low AGE diet, including 4-fold higher thiamin, 30% higher pyridoxine and 75% higher iron levels.

Another factor to consider is that frying above temperatures of 200°C results in oxidative lipid modification, polymerization and cyclization reactions of both the frying oil and the lipids present in food (8). Thus, the lipid oxidation and degradation products generated by excessive heating may also have contributed to the negative health effects observed by Stirban *et al.* (3).

Finally, it is interesting to note that this study (3) again contains data suggestive of low CML bioavailability. A dose of 15,100 kU of CML in the test meal resulted in a peak plasma CML concentration of approximately +4.5 U/mL four hours after the ingestion of the test meal. From this, it can be calculated that approximately 22.5 kU of CML were absorbed from this meal, or a mere 0.15%.

In conclusion, when designing human (or animal) AGE intervention trials, it is essential to add the AGEs to an identical basal diet with, as much as possible, identical and adequate micronutrients, vitamins and trace elements to enable the tracing back of any

observed effects to the presence of AGEs and not to other factors not controlled for. In addition, it is also essential to determine AGEs and protein and lipid oxidation markers other than CML since CML is i) not well absorbed from food and ii) also formed from lipid peroxidation that is increased during excessive thermal treatment of the food.

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Glyoxalase I: its impacts on anxiety-related and depression-like behaviors, and autism

by Toshio Miyata

Glyoxalase I is a cytosolic enzyme present in all cells and tissues. It is responsible for glutathione-dependent detoxification of alpha-oxoaldehydes (e.g. methylglyoxal, glyoxal), which are involved in the formation of advanced glycation end products (AGEs) and has thereby been implicated in the AGE accumulation-related human disorders (e.g., diabetic and uremic complications and atherosclerosis). Indeed, a glyoxalase I-deficient human or glyoxalase I-overexpressing mice/rats are associated with an increased or decreased tissue AGE formation, respectively: the former patient suffered from recurrent events of cardiovascular diseases (1).

Recent genetic and animal studies suggested an interesting link from glyoxalase I gene expression to behavior and mental biology (2). Hovatta et al. first demonstrated an association between glyoxalase I gene expression and anxiety-related behavior in mice (3). The validity of glyoxalase I expression as a possible biomarker of anxiety-related behavior has been investigated and confirmed by subsequent animal studies: glyoxalase I level is a determinant of anxiety-related behavior in high (HAB) and low (LAB) anxiety rats and mice (4). Also in humans, Ditzen et al showed that glyoxalase I levels are reduced significantly in patients suffering from anxiety disorders as compared to mentally healthy controls (5).

The possible involvement of this gene has also been shown in another human neuron-developmental disability, namely, autism which is a childhood disorder characterized by deficits in verbal communications, impairments in social interactions, and repetitive behaviors. Proteomic studies by Junaid et al identified a single nucleotide polymorphism in glyoxalase I as an autism susceptibility factor (6). They further revealed, by biochemical and immunochemical measurements, a 38% decrease in glyoxalase I enzyme activity and an accumulation of AGEs in brains from autism patients.

These data may provide exciting avenues for glyoxalase I biology in behavior and mental science. Still, the molecular mechanisms underlying these pathologies remain completely elusive. It remains unknown whether the altered glyoxalase I expression is an active contributor to the pathogenesis or is a mere metabolic result of neuron-developmental disability. Also unknown is the involvement of alpha-oxoaldehydes (e.g., methylglyoxal) and their end products (e.g., AGEs) in these disorders. Further research at molecular, biochemical levels is definitely necessary for the translation of these basic and animal data into diagnostic and therapeutic benefits in clinical medicine.

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Potential role for AGEs in cancer biology: a largely unexplored field of research

By Casper G. Schalkwijk

Due to lack of oxygen, tumours primarily rely on the anaerobic metabolism of glucose, a phenotype that was first noted by Otto Warburg in 1931. To compensate for this inefficient energy supply, tumours show a higher rate of glucose uptake and glycolysis. In fact, the increased level of glycolysis within tumour cells led to the discovery that glucose could be used as a possible tracer for the identification of tumour cells within the body. ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) is the most commonly used and available tracer used in Positron emission tomography (PET) as an imaging tool. After the transport of ¹⁸F-FDG across the cell membrane it is phosphorylated and trapped intracellularly and is resistant to further metabolic processes. With the increased numbers of glucose transporters on the tumour cell membrane allowing an increased uptake of ¹⁸F-FDG, there is a gradual accumulation of ¹⁸F-FDG in these malignant cells, allowing a three-dimensional image of the tumour with a PET scan.

¹⁸F-FDG PET not only images but also predicts tumour differentiation and outcome as recently demonstrated in hepatocellular carcinoma (1). Furthermore, there is also evidence to suggest that glucose uptake is increased with worsening grade of malignancy (2). This raised the question whether glucose uptake plays a role in malignancy and, if so, what is the underlying mechanism? One consequence of increased glucose uptake and glycolysis is the increased intracellular formation of advanced glycation endproducts (AGEs), most probably due to the formation of methylglyoxal. Methylglyoxal is a reactive dicarbonyl compound that is produced as a side-product during the glycolysis and is believed to be an important precursor in the formation of AGEs. The formation of AGEs has been linked to several detrimental processes associated with ageing, Alzheimer's disease, atherosclerosis, diabetes and heart failure, but, surprisingly enough, the presence of AGEs or their putative biological consequences in tumours is still largely unexplored. This is at least surprising because several reports demonstrated that the expression of the receptor for AGEs (RAGE) is closely associated with metastasis in various human cancers. In addition, a recent report demonstrated that the expression of RAGE is associated with angiogenesis in human oral squamous cell carcinoma (OSCC) (3). But are AGEs ligands for RAGE in tumours? We do not know. Although in the aforementioned study, the effect of HMGB1 as a RAGE ligand was studied in relation to the expression of VEGF and VEGF-C in human OSCC cell lines, it has previously been demonstrated that there is a difference in VEGF induction between AGE and HMGB1 (4) with AGE–bovine serum albumin, with a more pronounced effect on VEGF expression than HMGB1 in colorectal cancer cell lines.

These reports and a very recent report about an early increase of AGEs in breast cancer followed by a further increase of AGEs in patients with progressive disease (5) warranted further studies about a putative role of AGEs in tumour biology.

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Maillard reaction and the Creutzfeldt-Jakob disease variant

By Rosario Zamora

Prion diseases or transmissible spongiform encephalopathies are neurodegenerative disorders that have been described in humans as Creutzfeldt-Jakob disease, in sheep and goats as scrapie, and in cattle as bovine spongiform encephalopathy (BSE). BSE first appeared in the United Kingdom after 1986. It appears that the cause of the BSE outbreak was feeding misfolded prion protein-contaminated meat and bone meal (MBM) acquired from rendered carcasses of BSE- or scrapie-infected ruminants to healthy cattle. It is thought that the recycling of BSE-infected bovine tissues augmented the concentration of misfolded prion protein in commercial MBM, thus causing the subsequent BSE epidemics. Therefore, in order to ensure that by products such as MBM can be safely used in the future, the development of a technology that can be used for treating large amounts of by products at low costs is required.

A recent study by Suyama et al. (1) has suggested the use of the Maillard reaction to inactivate prions. They found that the Maillard reaction employing a formulation of glucose in combination with sodium hydrogen carbonates effectively reduced the infectivity (approximately 5.9-log reduction) of a scrapie-infected hamster brain homogenate. In addition to a bioassay, a protein misfolding cyclic amplification technique was also used as a rapid test for assessing misfolded prion protein inactivation. This assay also indicated that the misfolded prion protein in the infected material significantly decreased following exposure to Maillard reaction conditions. Therefore, the Maillard reaction has been suggested for the decontamination of large quantities of by products such as MBM.

Although additional studies are needed to validate the glucose formulation as a routinely applicable reprocessing procedure for prion decontamination, this study presents a new contribution to the potential use of Maillard reaction to increase food safety, which has also been suggested, for example, in the inactivation of food allergens (2).

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Postprandial hyperglycemia and inflammatory signaling

By Timo Buetler

Based on earlier reports on postprandial increases in NF- κ B activation and inflammatory signals (1), Schiekofer *et al.* (2) reported last year that postprandial mononuclear NF- κ B activation was increased by a single meal of casein independently of the AGE content of the casein meal. For this study, the authors prepared a high and a low AGE meal by heating casein (40h, 50°C) either with glucose (high-AGE meal) or with sorbitol (low-AGE meal). Nine healthy volunteers ate 250g of modified casein in a cross-over design after an overnight fast. The two meals were separated by a 2 week wash-out period. The high-AGE meal contained 3-times as much CML as the low-AGE meal and pentosidine was only detected in the high-AGE meal. As with earlier studies, eating of the high CML casein did not result in a significant increase in plasma CML levels. The results show that NF- κ B was significantly activated after the intake of either meal and was, thus, independent of the AGE content. In another recent article Schiekofer *et al.* (4) reported that the high NF- κ B activity in type 1 diabetic patients can be reduced by controlling glucose levels with insulin.

This work adds to a long list of reports showing that ingestion of a meal increases reactive oxygen species (ROS) and inflammatory signals (1). While part of this response may be due to postprandial hyperglycemia, a number of articles have reported that high fat meals can also induce ROS and inflammatory signals (3). Together, these reports show that the increase in blood glucose, lipids and triglycerids from the ingestion of a single meal, even in healthy individuals, can increase ROS generation and markers of inflammation. The latest report by Schiekofer *et al.* (2) now shows that this effect does not appear to be mediated by AGEs.

What does this mean for AGEs in the diet relative to lipids and glucose? It appears that the main mediators of potential adverse health effects (free radicals, inflammation, and vascular dysfunction) are rather lipids and glucose than AGEs. Both lipids and glucose, and probably the breakdown products of both generated during heating of food, will result in AGE formation and, potentially, in adverse health effects and it is unlikely that the AGEs present in food cause adverse health effects.

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Interpretation of elevated plasma and tissue AGE contents: recommendation for GFR estimates

By Toshio Miyata

Advanced glycation end products (AGEs) increase with age and accumulate at a faster rate in diabetes mellitus as a consequence of hyperglycemia. AGEs also rise in patients with renal dysfunction and failure, irrespective of the presence or absence of diabetes, probably as a result of an increased generation (due to an enhanced oxidative stress) or of a decreased removal (detoxification or clearance) of carbonyl precursors of AGEs.

Renal function is an independent and critical determinant of plasma and tissue AGE content. Estimation of renal function, namely, glomerular filtration rate (GFR), is therefore recommended for precise interpretation of serum AGE levels. Unfortunately, serum creatinine (Cr) and urea nitrogen levels are not appropriate markers to evaluate moderately deteriorated GFR: both are influenced by several factors other than GFR and they become abnormal only at an advanced stage of renal failure (GFR, ~30 ml/min).

Chronic kidney disease (CKD) is defined by either a GFR of less than 60 ml/min or the presence of histological, radiological and biochemical kidney damage, regardless of the cause, for three or more months. It is a recently recognized public health problem: in Japan only, over nineteen millions subjects have a GFR below 60 ml/min. A more convenient, precise estimate of GFR has thus been proposed to facilitate early detection of renal deterioration. It relies on an equation based on serum Cr, and has been recommended as a tool in a campaign to educate physicians, patients, and the public worldwide (1, 2).

Among equations proposed so far, the Modification of Diet in Renal Disease (MDRD) study (below) and Cockcroft-gault equations are mostly utilized and recommended.

The MDRD equation: estimated GFR = $186 \times [\text{Serum Cr (mg/dl)}]^{-1.15} \times (\text{age})^{-0.203}$ (x 0.742 for female).

MDRD GFR calculator is available on the web of National Kidney Foundation (http://www.kidney.org/professionals/kdoqi/gfr_calculator.cfm). In the near future, GFR estimated using this equation based on serum Cr will be reported automatically from clinical laboratories.

Example: An 80 year old non-diabetic female with serum Cr of 1.2 mg/dl. Conventional dipstick in spot urine specimen reveals no obvious proteinuria. Nevertheless, the MDRD equation estimates her GFR as 46 ml/min, a value pointing to a moderate renal dysfunction (Stage 3 in CKD classification). Given a slightly elevated serum pentosidine or other AGE moieties in this patient, it cannot be attributed merely to ageing but rather may reflect the moderate renal dysfunction. GFR estimates may thus allow us to take into consideration of possible contribution of renal dysfunction.

Despite some limitations of current GFR estimates (imprecision at high GFR, racial difference, extremes of age and body size, etc), GFR estimates provide a convenient and more precise determination of renal function to be utilized in the interpretation of plasma and tissue AGE levels.

Of note, factors initiating or aggravating CKD include ageing and diabetes, both of which impact on AGE genesis. Assessment of the respective contributions of moderate renal dysfunction and ageing (or diabetes) warrants a cautious approach. CKD is also an independent risk factor for cardiovascular diseases (CVD). It is therefore not surprising that AGEs (biomarkers affected largely by renal function and thereby by the presence of CKD) emerge as a useful surrogate biomarker for CVD.

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Detection of soluble forms of RAGE: what does it tell us?

by Katrien Gaens and Casper G. Schalkwijk

The receptor for advanced glycation endproducts (RAGE) is composed of three extracellular immunoglobuline-like regions, a transmembrane domain, and a cytosolic tail. This receptor, which is expressed in many cell types, interacts with different families of ligands that mediate diverse functions. Upregulation of RAGE is involved in the pathogenesis of many chronic diseases such as diabetes, atherosclerosis and neurodegenerative disorders and is associated with tissue injury and vascular complications.

Soluble forms of RAGE were previously shown to appear in human circulation. The family of soluble RAGE consists of splice variants of RAGE and proteolytically cleaved and shed forms of RAGE. esRAGE is an endogenous splice variant of RAGE lacking the transmembrane domain of the receptor resulting from alternative splicing of RAGE mRNA (1). In addition to esRAGE, at least three different splice variants of RAGE have been described (2). The question remains how to explain the different expression patterns of transcripts regulated by the same promoter. The identification of regulators affecting the alternative splicing of RAGE pre-mRNA should be addressed in future studies. sRAGE is described as proteolytically cleaved forms of RAGE which are most probably shed into the bloodstream by action of extracellular metalloproteinases.

But what do these soluble forms of RAGE tell us? For the detection of soluble forms of RAGE, two immunoassays are commercially available. In the immunoassay of R&D systems (Minneapolis, MN, USA), antibodies are used that detect total circulating soluble forms of RAGE, i.e. esRAGE, other splice forms of RAGE and cleaved and shed forms of RAGE. The immunoassay of B-Bridge (B-Bridge International, Inc. CA, USA) used an antibody against a unique C-terminal 16 amino acid peptide that recognizes esRAGE only (3). It has been determined that the immunoassay of esRAGE by the B-Bridge assay is not subject to interference by AGE ligands for RAGE such as AGEs, the S100/calgranulin family of proteins of HMGB-1, indicating that this ELISA detects total free esRAGE. In contrast, as far as we are aware, it is not known whether the immunoassay of sRAGE of R&D systems also detect sRAGE complexed with one of their ligands. This latter should be investigated. A recent report measured both sRAGE and esRAGE in type 2 diabetic patients (4). esRAGE correlated significantly with total sRAGE, but were found to be ~5 times lower than total sRAGE.

With these two immunoassays, the significance of these soluble forms has been studied in relation to different aspects of metabolic and vascular disease. Previously published studies reported on the association of sRAGE and esRAGE with diabetes, vascular complications and metabolic control. Some inconsistent results were noted. For instance, one study detected increased levels of sRAGE (5), while another study described decreased levels of esRAGE in patients with type 1 diabetes (6). But are these data inconsistent? It seems more likely that esRAGE and sRAGE are distinct markers with an appearance in the circulation due to different mechanisms: esRAGE a consequence of RAGE mRNA processing while the detection of sRAGE is a sum of all the soluble RAGE, other splice variants of RAGE and shed forms of RAGE.

Indeed, the recent study of Humpert et al (4). argues for a distinct role of sRAGE and esRAGE as potential markers in diabetes: while total sRAGE is associated with 24 hour albumin excretion i.e. microvascular damage, plasma esRAGE is not associated with any markers of disease. Neither sRAGE nor esRAGE were associated with macrovascular disease in this study. On the other hand, sRAGE are independently associated with the presence of coronary artery disease in non-diabetic men (7) and low circulating plasma

esRAGE is a predictor of cardiovascular mortality in a cohort of patients with end-stage renal disease (8).

It is obvious that further clinical studies are required to elucidate the impact and potential relevance of all the soluble forms of RAGE as valuable biomarkers and/or risk factor of cardiovascular complications.

More important, we should realize that there are major differences between the two different commercially available assays. These differences are of importance for the accurate interpretation of results from plasma measurements in clinical studies.

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New applications for old reactions

By Rosario Zamora

As evident from the recipes in cookery books down through the ages, the Maillard reaction has been employed to enhance the sensory qualities of many dishes in relation to both aroma and browning development. However, these reactions are unavoidable consequences of the close contact of carbohydrates (or oxidized lipids) and amino compounds, and they are produced to some extent even when they are unwanted or when temperature is relatively low. Therefore, different attempts have been carried out to find potential applications for these reactions in these latter cases. Two recent articles may be good examples of new applications for these old reactions.

In the first of them, Pilin et al. (1) have studied the changes of color of different human tissues as a marker of age. Age estimation in forensic medicine is an important procedure in the identification of an unknown dead body, and there are methods of age estimation according to teeth or skeleton. However, little attention was paid until now to the soft tissues. This study examined age-related color changes in tissues that included intervertebral discs, Achilles tendons and rib cartilage. These color changes seem to be a consequence of the accumulation of AGEs during life which result in the protein-bound brown and fluorescent adducts. The degrees of color change were different in different tissues. They were well correlated with age in the rib cartilage and also in the intervertebral discs. On the other hand, nearly no relation between color and age was found in the Achilles tendon. The study concludes that reliable age estimation based on color changes may be performed up to the age of 45 especially from the rib cartilage where color changes are most evident. After 45 years of age, the spread of values increased and the reliability of the developed method was low. Although there is no explanation for this limit of age, it might be related to the contribution of disease – as discussed below – to these color changes.

It is well-known that certain diseases also contribute to the accumulation of AGEs and, therefore, to the development of protein browning and fluorescence. A recent example of the potential application of this phenomenon, is the use of skin autofluorescence as a predictor of cardiac mortality in diabetes. Meerwaldt et al. (2) have continued their studies on the use of skin autofluorescence, previously also applied to the prediction of mortality in hemodialysis patients (3, 4), to the prediction of cardiac mortality in diabetes. They measured skin autofluorescence in 48 type 1 and 69 type 2 diabetic patients and 43 control subjects. They found that autofluorescence correlated with mean haemoglobin A1C, triglycerides, and LDL. Autofluorescence values further increased with age, microalbuminuria, dialysis treatment, and diabetes duration. They concluded that autofluorescence was strongly related to the presence of coronary heart disease and predicted mortality, and, therefore, it may provide important clinical information for risk assessment.

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Letter to the Editor

By Timo Buetler

Response to Pischetsrieder, *IMARS Highlights* (2007) Vol. 2, no. 2, p. 15.

The paper (1) discussed in the last *IMARS Highlights* indeed is very interesting as it shows that protein extracts from inflamed colon tissue from patients suffering from Crohn's disease or colitis contain fractions that can induce inflammation in recipient tissues or cells. The paper shows convincingly that this response is to a large degree mediated by RAGE as the response is absent in RAGE deficient mice or can be suppressed by soluble RAGE in cell cultures. Thus, the active fraction very likely contains RAGE ligand(s).

Unfortunately, the authors (1) may have been somewhat biased towards AGEs as the ligands responsible for RAGE activation. In fact, the data do not allow the conclusion that CML or protein glycation is responsible for mediating the observed effects since the active fractions always contained both CML-modified proteins and the known pro-inflammatory RAGE ligand S100A8/A9. In order to test whether glycation or S100A8/A9 were responsible for the observed NF- κ B activation, it would have been necessary to separate S100A8/A9 from the rest of the glycated proteins. This has in fact been done and is shown in figure 5 (1). The data presented show that the flow-through of the S100A8/A9 affinity column that was depleted of S100A8/A9 but presumably still contained all the other glycated proteins present in the extract was unable to stimulate NF- κ B activation (figure 5B). All the NF- κ B activation capacity of the inflamed tissue extracts was retained by the S100A8/A9 affinity column, thus showing that S100A8/A9 and not glycation *per se* was the mediator of NF- κ B activation and inflammation.

Because the data presented appear to show that all S100A8/A9 protein was glycated the important question now is how glycation affects the S100A8/A9-mediated NF- κ B activation. Finally, it should be noted that the evidence for CML being a RAGE ligand is rather weak (2-4) and thus it is unlikely that CML was capable of transmitting inflammatory activity.

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